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Proto-oncogene Expression in Human Breast Cancer

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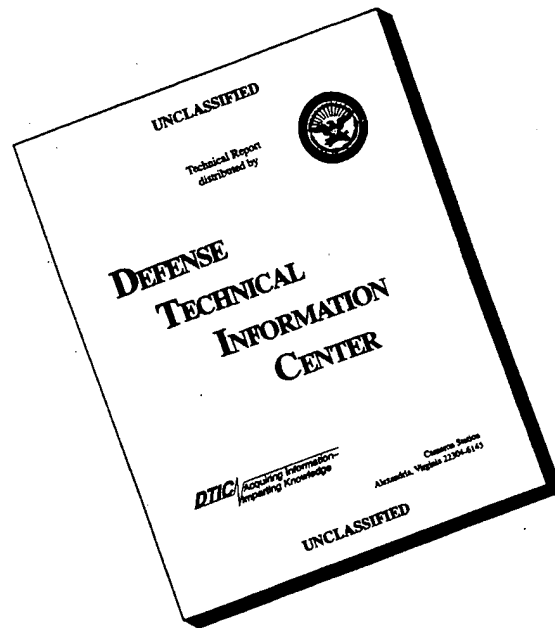
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## INTRODUCTION

The objective of this research is to examine regulation of the c-myc proto-oncogene in normal and transformed cells. C-myc has been shown to be overexpressed in a number of human cancers; identifying factors that control c-myc expression and characterizing the mechanisms by which they function should provide insight into how these factors affect normal and transformed cell growth.

In vivo, DNA is complexed with histones and nonhistone proteins to form chromatin, which in general, is refractory to gene expression. Thus to understand how genes such as c-myc become activated, it is necessary to identify and characterize both the in vivo structural state of the promoter controlling gene expression as well as factors that can play a role in relieving chromatin mediated inhibition and facilitating transcription. Previous work has identified and partially characterized a cellular protein complex, called hSWI/SNF, that can rearrange chromatin structure in an ATP dependent manner and increase the ability of transcription factors to bind to their recognition sequences when these sequences are incorporated into chromatin (Kwon et al, 1994; Imbalzano et al, 1994). In the present work, the mechanism of hSWI/SNF function has been investigated using in vitro reconstitution techniques. In addition, the function of hSWI/SNF in the stimulation of transcription elongation from the human hsp70 promoter has been examined. The hsp70 promoter, like the c-myc promoter, is regulated at both the level of transcription initiation and transcription elongation (Spencer and Groudine, 1990), and may therefore serve as a model gene for examining regulation at the level of transcriptional elongation.

## BODY

### I. SUMMARY

Much of the investigation over the past year has focused on characterizing the activity of the human SWI/SNF complex (hSWI/SNF), which was previously shown to rearrange chromatin structure in an ATP dependent manner as well as to facilitate transcription factor binding to nucleosomal DNA (Kwon et al, 1994; Imbalzano et al, 1994). This work has examined the mechanism of action of the hSWI/SNF complex and also determined that hSWI/SNF can increase the ability of a transcriptional activator protein to stimulate transcriptional elongation on nucleosomal DNA.

### II. EXPERIMENTAL DIRECTION

Understanding the mechanism of action of the hSWI/SNF complex is of central importance to analyzing regulation of c-myc and other gene expression. To date, the hSWI/SNF complex has been shown to alter chromatin structure and to facilitate binding of transcription factors to nucleosomal DNA (Imbalzano et al, 1994; Kwon et al, 1994), and individual components of the complex can enhance stimulation of transcription by nuclear hormone receptors in eukaryotic cells (Muchardt and Yaniv, 1993; Khavari et al, 1993) and can bind to the the retinoblastoma (Rb) oncoprotein (Dunaief et al, 1994; Singh et al, 1995). The interaction with Rb has been implicated in enhancing nuclear receptor stimulated transcription (Singh et al, 1995) and in causing arrest of the cell cycle (Dunaief et al, 1994). Another component of the complex has been shown to bind to HIV-1 integrase and stimulate DNA joining in vitro, suggesting that the component may promote integration of viral DNA into the genome (Kalpana et al, 1994). In addition, the complex and at least some of the component genes are evolutionarily conserved. Yeast SWI/SNF complex has similar nucleosome altering capabilities (Cote et al, 1994), and the yeast SWI and SNF genes are required for mating type switching and is required for the activation of many yeast genes (reviewed in Carlson and Laurent, 1994). Similarly, a Drosophila SWI homolog is required for activation of many homeotic genes and thus for proper development of the organism (Tamkun et al, 1992). Thus SWI/SNF and/or its components globally affect gene expression, chromatin structure, and cell growth and division, development and differentiation, and potentially are involved in or affect events leading to oncogenesis.

Before commencing work on a specific promoter like c-myc, our original goal was to complete a thorough characterization of hSWI/SNF mechanism of action and its role in transcription elongation on nucleosomal templates in vitro. This has taken longer than anticipated. In addition, other lines of experimentation developed that were not listed in the original proposal and that did not or have not yet yielded definitive results, such as whether purified Rb protein could affect nucleosome disruption by hSWI/SNF and whether transcriptional activators can target hSWI/SNF to promoter sequences. The work accomplished and presented here advances our understanding of how hSWI/SNF may function to make genomic DNA accessible to transcription factors and the general transcription

machinery during activation of transcription initiation and elongation. Future work will focus on applying these findings to c-myc expression.

### **III. PROGRESS ON SPECIFIC AIM #2: In vitro analysis of c-myc promoter occupancy and chromatin structure**

#### **A. Characterization of hSWI/SNF activity**

**1. Experimental Background** To characterize the mechanism of hSWI/SNF action, I have employed nucleosomal reconstitution techniques to create rotationally phased nucleosomes in vitro and have examined cleavage of nucleosomal DNA by DNase I as a measure of the ability of hSWI/SNF to alter nucleosome structure. DNase I can only cleave nucleosomal DNA at the point of the DNA helix that is farthest away from the histone core, or in other words, it cleaves the nucleosomal DNA once every turn of the helix, or approximately every 10 base pairs (bp) per strand. Because the assembled mononucleosomes are phased (*ie*- the DNA and the histone core that comprise the nucleosome are physically associated in exactly the same manner for every molecule in the population) and the DNA is end labeled with  $^{32}\text{P}$  on one strand only, limiting digestion of the mononucleosomes by DNase I results in the appearance of a cleavage ladder whose products are 10 bp apart (see Figure 1, lane 2). Addition of the hSWI/SNF complex in the presence of ATP results in a decrease in the intensity of the bands forming the 10 bp ladder and the appearance of novel cleavage products in between (see Figure 1, lanes 4-6). Thus the activity of hSWI/SNF alters the nucleosome structure in a manner that allows increased access to the DNase I enzyme.

#### **2. Results**

**a) hSWI/SNF activity is dependent on the presence of a hydrolyzable adenosine triphosphate (ATP).** Deoxy-adenosine triphosphate (dATP) could substitute for ATP, however, when dATP was used, a 10-fold higher concentration was required for nucleosome disruption to occur. The nonhydrolyzable ATP analogs ATP- $\gamma$ -S and AMP-PNP did not support nucleosome disruption, nor did adenosine diphosphate (ADP), nor any other nucleotide triphosphate (Figure 1).

**b) ATP is not continuously required for hSWI/SNF activity; the hSWI/SNF mediated alteration in nucleosome structure is stable.** If ATP were continuously required for activity, that is, if the structural change induced by hSWI/SNF were transient, and the nucleosome reverted to its original form after ATP mediated disruption, then removal of ATP from the reaction prior to DNase I cleavage should generate the same 10 bp ladder of cleavage products seen when untreated nucleosomes are digested with DNase I. Alternately, if the change in structure induced by hSWI/SNF is stable, then the altered DNase I digestion pattern should appear, even if ATP is removed from the reaction. When hSWI/SNF and nucleosomes were mixed in the presence of ATP and were subsequently exposed to apyrase, which cleaves ATP (Figure 2), or to a 200-fold



excess of ATP- $\gamma$ -S, which is sufficient to inhibit disruption (Figure 3), the altered DNase I digestion pattern was maintained, indicating that the change in nucleosome structure induced by hSWI/SNF is stable.

c) Nucleosome disruption by hSWI/SNF requires the simultaneous presence of nucleosomes and ATP.

To determine whether ATP was inducing a conformational change in hSWI/SNF structure that made it "active" and therefore unaffected by the removal of ATP, an order of addition experiment was performed in which hSWI/SNF was mixed with ATP, was subsequently treated with apyrase to remove ATP, and then was mixed with the nucleosomes. In this experiment, the 10 bp ladder of DNase I cleavage products was observed (Figure 4, lane 10), indicating that nucleosome disruption required the simultaneous presence of hSWI/SNF, ATP, and nucleosomes. This result suggests that the hydrolysis of ATP is required for altering the structure of the nucleosome and not for modifying the hSWI/SNF (eg- by phosphorylation, by altering conformation). This work does not exclude the possibility that ATP modifies hSWI/SNF structure, but it indicates that even if such a modification occurs, it is not sufficient for nucleosome disruption.

d) Facilitated binding of the GAL4-AH transcription factor to mononucleosomes containing a GAL4 binding site requires prior disruption by hSWI/SNF but does not require concurrent activity.

Previous work has shown that if the nucleosome contains a transcription factor binding site, treatment of the nucleosome with hSWI/SNF significantly increases the ability of the transcription factor to bind (eg- GAL4 derivatives) or facilitates binding where no binding was previously observed (eg- TATA binding protein) (Kwon et al, 1994; Imbalzano et al, 1994). Addition of apyrase to reactions containing nucleosomes and ATP prior to addition of hSWI/SNF prevented hSWI/SNF-mediated nucleosome disruption and prevented facilitated binding of GAL4-AH to the nucleosome (Figure 5- lanes 11-14). When apyrase was added to reactions containing nucleosomes and ATP after the addition of hSWI/SNF, nucleosome disruption was observed, and upon addition of GAL4-AH, facilitated binding of the transcription factor to the altered nucleosome was observed (Figure 5- lanes 15-18). These results indicate that the increased ability of the transcription factor to bind to nucleosomal DNA is not dependent on concurrent hSWI/SNF activity and that hSWI/SNF facilitated binding of transcription factors to nucleosomal DNA can be a multi-step process, where first the nucleosome structure is stably altered by hSWI/SNF, thereby facilitating subsequent interaction with the transcription factor.

e) hSWI/SNF may stably associate with the nucleosome.

The observation that hSWI/SNF stably alters nucleosome structure suggested that perhaps hSWI/SNF was stably associated with the altered nucleosome. Preliminary sedimentation studies indicate that when hSWI/SNF and nucleosomes are mixed and subsequently sedimented on a glycerol gradient, the nucleosomes have a significantly increased mobility in the gradient. This change in mobility is apparently not dependent on ATP, although it appears that the presence of ATP

slightly changes the increase in migration. Current efforts are designed to show that the increase in sedimentation is due to a specific interaction between hSWI/SNF and the nucleosome, and to determine the effect of ATP on the interaction.

## **B. Examination of the ability of hSWI/SNF to stimulate transcription elongation from a nucleosomal human hsp70 promoter.**

**1. Experimental Background** In collaboration with Steven Brown, a graduate student in the lab, investigations into the role of hSWI/SNF in transcription elongation in chromatin have been performed (Brown et al, submitted). Steven has shown that in vivo, there is a paused, transcriptionally engaged RNA polymerase II molecule associated with the human heat shock protein 70 (hsp70) promoter, even in the absence of heat shock, when the locus is transcriptionally inactive (Brown et al, Figure 1). To mechanistically examine how activation of the hsp70 promoter occurs upon heat shock, Steven has recreated the unactivated promoter in vitro, establishing a nucleosomal template that has a paused, transcriptionally engaged RNA polymerase molecule and a nascent RNA transcript of the same length that appears in vivo (Brown et al, Figure 2A).

## **2. Results**

### **a) Human heat shock factor activation domain and fractions containing hSWI/SNF cooperate to increase transcriptional elongation on nucleosomal human hsp70 promoters.**

The activator that stimulates hsp70 transcription in vivo is the heat shock factor (HSF). Addition of an activator that contains the HSF activation domains to the in vitro system provided a small increase in the amount of transcriptional elongation through the pause (fusion proteins consisting of the HSF activation domains and the GAL4 DNA binding domains were used in these experiments because the magnesium and detergent requirements to maintain native human HSF isolated from heat-shocked nuclei active are not compatible with nucleosome assembly conditions). When hSWI/SNF was also added to the reaction, there was a significant increase in the amount of transcriptional elongation, suggesting that addition of a factor able to disrupt nucleosome structure facilitated HSF activation of transcriptional elongation. There was no effect on elongation through the pause by hSWI/SNF in the absence of activator (Brown et al, see Figure 4A). Activators containing just the GAL4 DNA binding domain had a minimal effect on elongation (Brown et al, Figure 4B). Quantification of elongated product at different hSWI/SNF concentrations is presented in Brown et al, Figure 5.

### **b) GAL4-HSF and hSWI/SNF do not affect elongation from the paused Polymerase on naked hsp70 DNA.**

Examination of elongation on naked hsp70 templates revealed some short-lived pause sites, however, these were unchanged in the presence of GAL4-HSF and/or hSWI/SNF (Brown et al, Figure 6).

#### **IV. PROGRESS ON SPECIFIC AIM #1: In vivo analysis of c-myc promoter occupancy and chromatin structure.**

Mapping of c-myc promoter structure was proposed by chemical or enzymatic modification of genomic DNA in vivo, with subsequent detection of DNA cleavage sites by amplification with ligation mediated polymerase chain reaction (LMPCR). Due to the focus on further characterization of hSWI/SNF, only a few attempts to initiate in vivo mapping of c-myc promoter structure have been made; no signal was observed, indicating that optimization of LMPCR reaction conditions and/or different LMPCR primers will be required.

Detailed LMPCR protocols have been obtained from Drs. Tre McPherson and Ken Zaret, who have successfully examined and mapped in vivo chromatin structure on the mouse liver serum albumin enhancer (McPherson et al, 1993). In addition, to simplify the experimental protocol, HeLa cells, which grow quickly and are easy to culture, will be used initially to perfect experimental conditions.

## CONCLUSION

The research indicates that hSWI/SNF, a protein complex that can alter chromatin structure and facilitate the interaction of transcription factors with nucleosomal DNA, stably alters chromatin structure in a manner that depends on the hydrolysis of ATP. In addition, the alteration of chromatin structure is separable from the facilitation of transcription factor binding, indicating that sequential steps can occur in the process by which inactive, inaccessible chromatin becomes transcriptionally activated. In addition, the results indicate that hSWI/SNF can increase transcriptional elongation in vitro on nucleosomal hsp70 promoters. This increase in transcription is dependent upon the presence of chromatin structure, as no increase is observed on naked DNA. This result suggests that hSWI/SNF is altering the chromatin structure on the reconstituted promoter and thereby increasing the ability of the transcription factors and RNA polymerase to function.

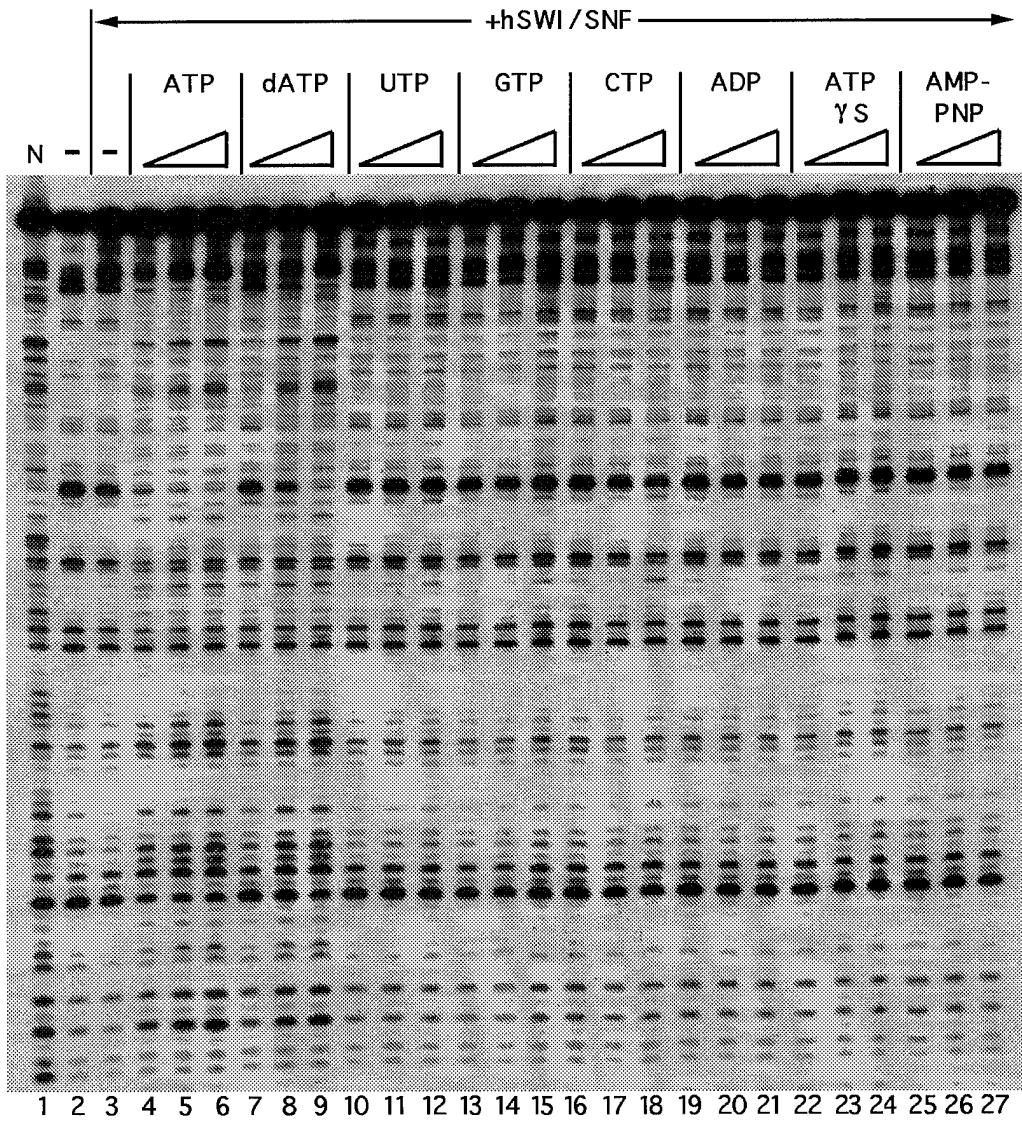
The work presented in this report advances our understanding of how a cellular factor, hSWI/SNF may function to make genomic DNA accessible to transcription factors and the general transcription machinery during activation of transcription initiation and elongation. Future work will focus on applying these findings to c-myc expression.

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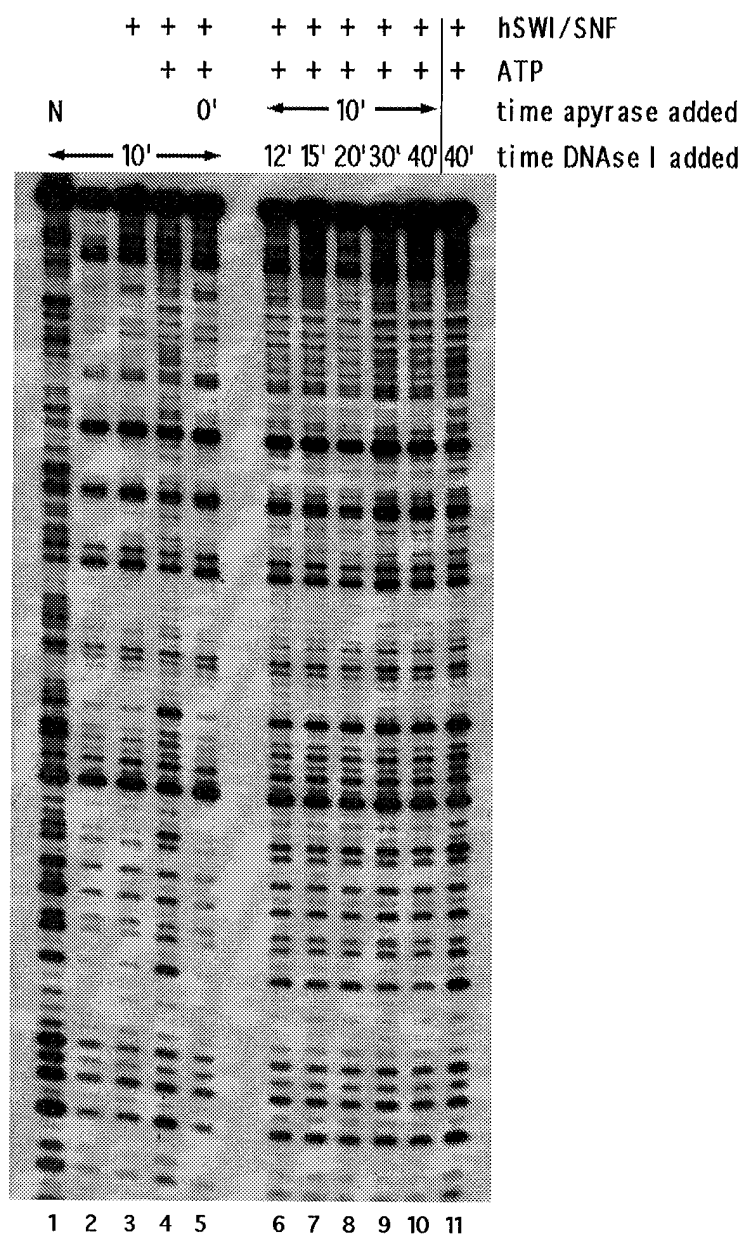
Figure 1



**Figure 1** ATP and dATP support nucleosome disruption by hSWI/SNF. Reactions contained 0.3 nanograms ( $1.2 \times 10^{-10}$  M) PH MLT nucleosomes labelled at the Eco RI site [approximately 3 nanograms ( $1.2 \times 10^{-9}$  M) total nucleosomes] in 25 microliter reactions containing 12 mM HEPES pH 7.9, 60 millimolar KCl, 7 millimolar  $MgCl_2$ , 15% glycerol, 0.5 micrograms BSA, 0.6 millimolar DTT, 0.06 millimolar EDTA and, where indicated, 600 nanograms of hSWI/SNF (lanes 3-27). Where indicated, reactions also contained 0.02 millimolar (lanes 4,7,10,13,16,19, 22,25), 0.2 millimolar (lanes 5,8,11,14,17,20,23,26), or 2 millimolar (lanes 6,9,12,15,18, 21,24,27) nucleoside tri- or di-phosphate or nonhydrolyzable ATP analog. Reactions were incubated at 30° C for 30 minutes, followed by DNase I digestion. N represents naked DNA.

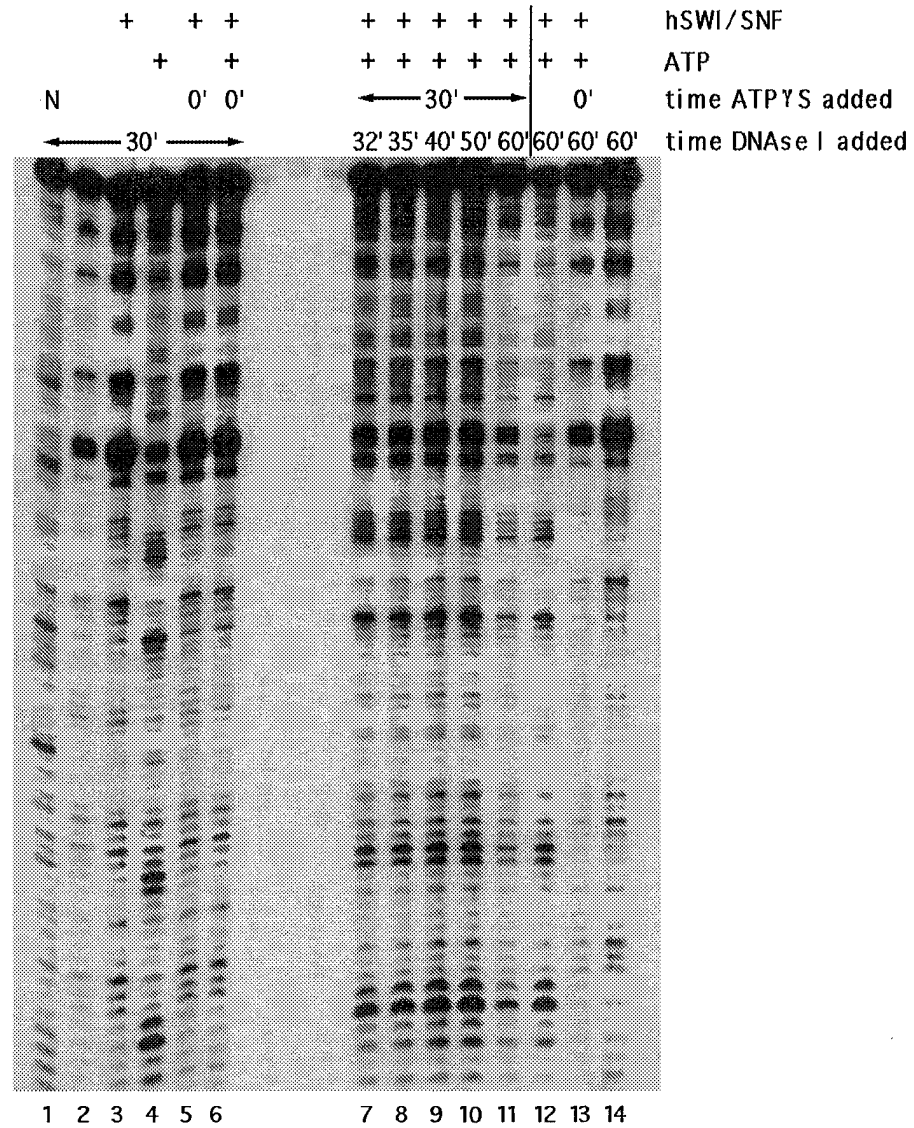


Figure 2



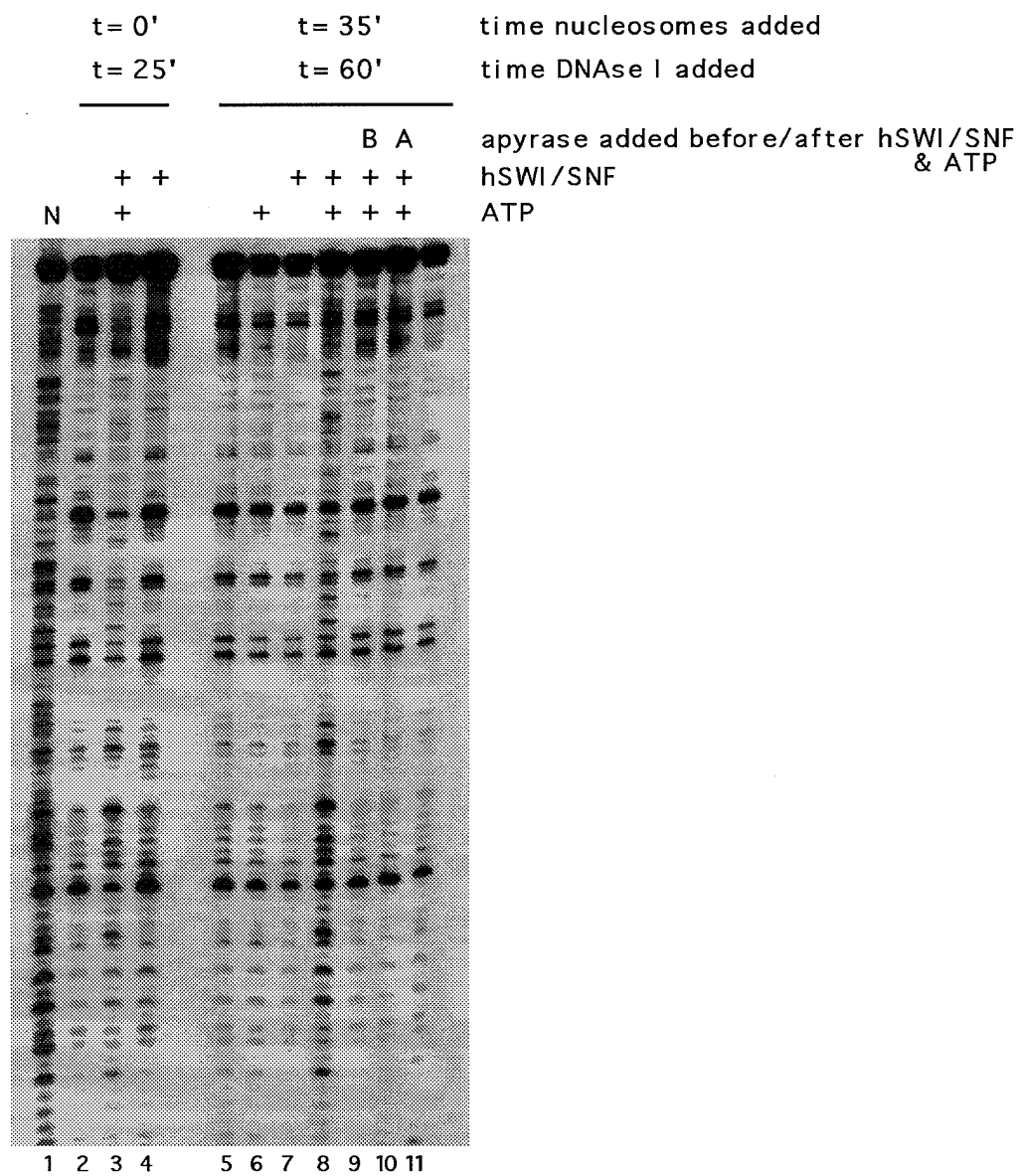
**Figure 2** Nucleosome disruption by hSWI/SNF is stable upon removal of ATP by apyrase. Reaction conditions were as described for Figure 1, except that where indicated, reactions contained 0.02 millimolar ATP and 300 nanograms hSWI/SNF "A". Nucleosome disruption as assessed by DNase digestion 10 minutes after addition of hSWI/SNF is seen in lane 4; disruption after 40 minutes is seen in lane 11. Addition of 1 U of apyrase prior to hSWI/SNF addition prevented nucleosome disruption (lane 5). Apyrase was added to identical reactions 10 minutes after addition of hSWI/SNF (lanes 6-10) and disruption was assessed by DNase I digestion 2 minutes (lane 6), 5 minutes (lane 7), 10 minutes (lane 8), 20 minutes (lane 9), or 30 minutes (lane 10) after addition of apyrase. Reaction start times were staggered such that reactions presented in lanes 6-11 were started 5 minutes after the reactions presented in lanes 1-5. N represents naked DNA.

Figure 3



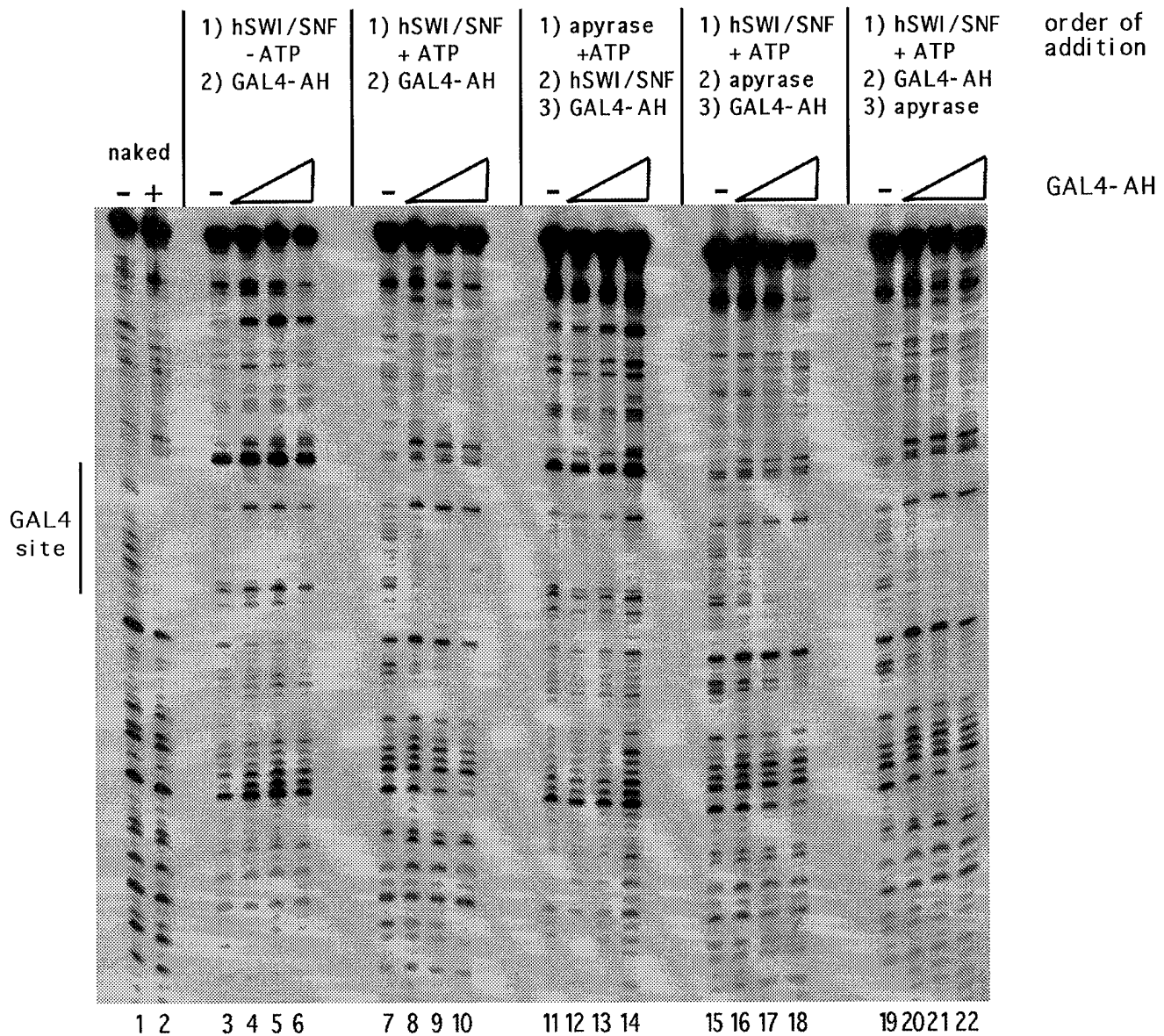
**Figure 3** Nucleosome disruption by hSWI/SNF is stable when hSWI/SNF activity is competitively inhibited by excess ATP $\gamma$ S. The experiment is similar to those presented in Figure 2 . PH MLT nucleosomes were labelled at the Bam HI end. Where indicated, reactions contained 600 nanograms of hSWI/SNF, 0.02 millimolar ATP, and 4 millimolar ATP $\gamma$ S (200-fold excess). ATP dependent nucleosome disruption 30 or 60 minutes after hSWI/SNF addition, as assayed by DNase I digestion, is seen in lanes 4 and 12. Substitution of excess ATP $\gamma$ S for ATP did not support nucleosome disruption (lane 5 and Figure 1). Addition of excess ATP $\gamma$ S to reactions containing ATP prior to addition of hSWI/SNF prevented nucleosome disruption when assayed 30 minutes (lane 6) or 60 minutes (lane 13) later. Excess ATP $\gamma$ S was added to identical reactions 30 minutes after addition of hSWI/SNF (lanes 7-11) and disruption was assessed by DNase I digestion 2 minutes (lane 7), 5 minutes (lane 8), 10 minutes (lane 9), 20 minutes (lane 10), or 30 minutes (lane 11) after addition of ATP $\gamma$ S. N represents naked DNA.

Figure 4



**Figure 4** Nucleosome disruption by hSWI/SNF requires the presence of nucleosomes and ATP. Reactions were as described in Figure 1. PH MLT nucleosomes were labelled at the Eco RI end. Where indicated, reactions contained 600 nanograms of hSWI/SNF and 0.02 millimolar ATP. In the control reactions (lanes 2-4), ATP dependent nucleosome disruption is seen upon DNase I digestion 25 minutes after the start of the reactions (lane 3). For the reactions shown in lanes 5-11, reactions were initiated in the absence of nucleosomes. 1 U of apyrase was added to the reaction prior to the addition of hSWI/SNF (lane 9) or 25 minutes after addition of hSWI/SNF (lane 10). PH MLT nucleosomes were added to each reaction (lanes 5-11) 35 minutes after the start. Nucleosome disruption was analyzed by DNase I digestion 60 minutes after the start of the reactions. No disruption was observed in when apyrase was added after hSWI/SNF but before the nucleosomes (lane 10), indicating a requirement for the presence of nucleosomes for hSWI/SNF-mediated nucleosome disruption. N represents naked DNA.

Figure 5



**Figure 5** Facilitated binding of GAL4-AH to PH GAL4<sub>1</sub> mononucleosomes requires prior disruption by hSWI/SNF but does not require concurrent hSWI/SNF activity. Reaction conditions were as described for Figure 1. Where indicated, reactions contained 600 nanograms hSWI/SNF, 4 millimolar ATP, and no GAL4-AH (lanes 3,7,11,15,19),  $1 \times 10^{-7}$  molar GAL4-AH dimer (lanes 4,8,12,16,20),  $1 \times 10^{-6}$  molar GAL4-AH dimer (lanes 5,9,13,17,21), or  $1 \times 10^{-5}$  molar GAL4-AH dimer (lanes 6,10,14,18,22). Reactions were treated with hSWI/SNF in the absence (lanes 3-6) or in the presence (lanes 7-10) of ATP for 30 minutes and were then assayed by DNase I digestion. Alternately, 1 U of apyrase was added prior to hSWI/SNF addition (lanes 11-14); no facilitated GAL4-AH binding was subsequently observed. 1 U of apyrase was added to the reactions presented in lanes 15-18 30 minutes after addition of hSWI/SNF; 10 minutes later, GAL4-AH was added for 30 minutes, followed by DNase I digestion. Reactions presented in lanes 19-22 were identical to those in lanes 15-18, except that apyrase was added after the 30 minute exposure to GAL4-AH, followed by DNase I digestion 10 minutes later. For comparison, naked PH GAL4<sub>1</sub> DNA (lanes 1-2) was incubated for 30 minutes at 30° C, followed by addition 30 minute incubation in the absence (lane 1) or presence (lane 2) of  $2 \times 10^{-8}$  molar GAL4-AH. Samples were then digested with DNase I.



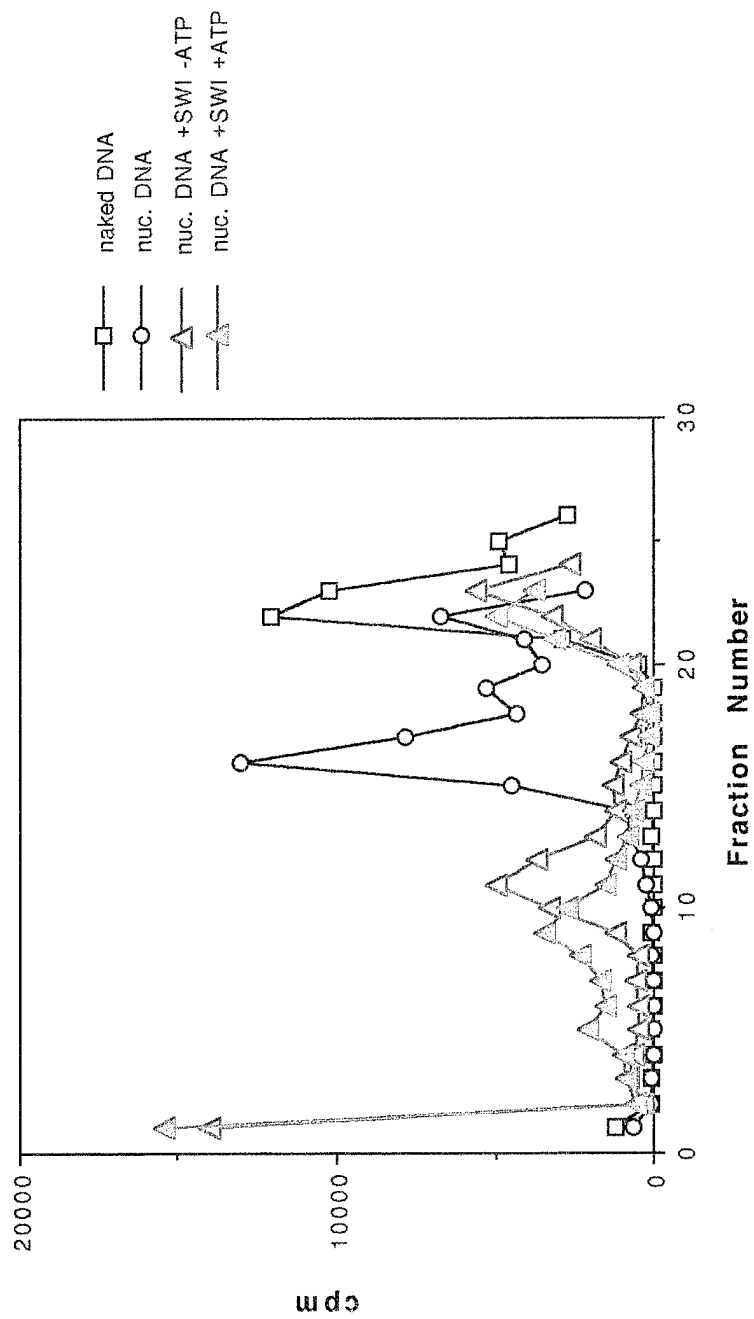


Figure 6

Figure 6 Glycerol gradient centrifugation of hSWI/SNF and PH MLT mononucleosomes. Shown is a representative individual experiment. Reactions were initiated as described in Figure 1 in the presence or absence of 4 millimolar ATP, except that they were scaled up 6-fold for all components. Following a 30 minute incubation at 30° C, the 150 microliter reactions were layered onto a 10-40% glycerol gradient and spun in a Beckman 55.1 rotor at 50000 rpm at 4° C for 4.5 hours. Three drop (~200 microliters) fractions were collected on ice. Each fraction was counted by Cerenkov counting in an LKB scintillation counter, and the counts per fraction was plotted. Fraction 1 represents the bottom of the empty gradient tube.

Appendix I

**Activator-dependent regulation of transcriptional pausing on nucleosomal  
templates**

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## **SUMMARY**

Promoter-proximal pausing during transcriptional elongation is an important way of regulating many diverse genes, including human *c-myc* and *c-fos*, some HIV genes, and the *Drosophila* heat shock loci. To characterize the mechanisms that regulate pausing, we have established a system that recapitulates in vitro the regulation of transcriptional elongation that we have observed on the human *hsp70* gene in vivo. We demonstrate that nucleosome formation increases by over 100-fold the duration of a transcriptional pause on the human *hsp70* gene in vitro at the same location as pausing is observed in vivo. Readthrough of this pause is increased by an activator that contains the human heat shock factor 1 transcriptional activation domains. Maximal effect of the activator requires that the system be supplemented with fractions that have hSWI/SNF activity, which has been shown previously to alter nucleosome structure. No significant readthrough is observed in the absence of activator, and neither the activator nor the hSWI/SNF fraction affected elongation on naked DNA; therefore, these results suggest that an activator can cause increased readthrough of promoter-proximal pausing by decreasing the inhibitory effect of nucleosomes on transcriptional elongation.

## INTRODUCTION

Mechanisms of gene regulation at the level of transcriptional elongation have been well-characterized in prokaryotes. Eukaryotic counterparts to these mechanisms are just beginning to emerge. Examples of regulated elongation in mammalian cells include the *myc*, *myb*, *fos*, *mos*, and *ada* genes, which exhibit a regulatory block to transcription near their 5' ends (reviewed in Spencer and Groudine, 1990). The *Drosophila hsp70* gene is also regulated at the level of elongation (Gilmour and Lis, 1986; Rougvie and Lis, 1988; Giardina and Lis, 1993). A paused, transcriptionally-engaged RNA polymerase ternary complex has been found over a relatively narrow promoter-proximal region on *hsp70* (Rasmussen and Lis, 1993). In response to heat shock, not only does the rate of initiation increase, but the transit time of polymerase through the pause is drastically reduced. Similar pauses have been found in *Drosophila* on other heat-shock genes (e.g. *hsp26*), metabolic genes (*gadph-1* and *gadph-2*) and structural genes ( $\beta$ 1-tubulin), so this phenomenon may be relatively widespread (Rougvie and Lis, 1990).

What causes and what releases a eukaryotic pause remains mostly a mystery. Pausing on HIV-1 is at least partially determined by an RNA-encoded TAR element and partially relieved by the Tat transactivator (reviewed in Cullen, 1990). On human *c-myc* (Krumm et al., 1995) and *Drosophila hsp70* (Lee et al., 1992) no such discrete elements have been found. Nonetheless, in vitro transcription studies on chromatin templates have shown that nucleosomes greatly enhance sequence-specific pausing (Izban and Luse, 1991). This enhanced pausing might be a consequence of the need for nucleosomes to be displaced during the transcription process (Studitsky et al., 1994; Clark and Felsenfeld, 1992). Hence it is plausible that nucleosomes might play a role in the control of eukaryotic transcriptional elongation by causing specific, regulatable pauses. These pauses might then be regulated by transcriptional activators, by elongation factors like TFIIF (Flores et al., 1989) and TFIIS (Reinberg and Roeder, 1987; Reines et al., 1989), or by chromatin-reorganizing factors like the SWI/SNF complex (Winston and Carlson, 1992;

Imbalzano et al., 1994; Kwon et al., 1994; Cote et al., 1994; Cairns et al., 1994) or the Drosophila NURF complex (Tsukiyama et al., 1995).

Activators have been implicated primarily in regulating transcriptional initiation on both naked and nucleosomal DNA, and current evidence suggests that they achieve their effects in several different ways. Direct contacts between activators and components of the general transcription machinery have been proposed to regulate pre-initiation complex formation and DNA melting at the promoter (reviewed in Kingston and Green, 1994; Tijan and Maniatis, 1994; Ptashne and Gann, 1990).

Studies on chromatin templates show that activators can relieve nucleosomal inhibition of transcription, possibly through contacts with the general transcription machinery and/or with complexes like SWI/SNF that directly destabilize chromatin structure (reviewed in Workman and Buchman, 1993). Finally, activators may also play a direct role in the regulation of elongation. In vivo, Yankulov et al. (1994) demonstrated that a variety of activators are able to stimulate elongation through pausing and termination sites on stably transfected reporter constructs, and Krumm et al. (1995) found that enhancers can increase readthrough of promoter-proximal pausing.

This paper provides evidence that the human HSF1 transcriptional activation domains can regulate elongation through the human *hsp70* promoter in vitro, and suggests that this is accomplished by overcoming a nucleosome-dependent block to transcriptional elongation. Specifically, we have developed an *in-vitro* system that mimics the regulation of transcriptional pausing on the human *hsp70* gene, and we show that this pause is dramatically increased by the presence of nucleosomes. The pause is released when reactions contain the HSF1 activation domain as part of a GAL4 fusion protein, and maximal release also requires fractions with hSWI/SNF activity. Neither GAL4-HSF nor the hSWI/SNF fraction affect elongation on naked templates, suggesting that their effects on elongation are specific to the nucleosome-dependent block. Hence, the data here imply that nucleosomes may play a role in the promoter-proximal pausing observed on

numerous genes in vivo, and that an important aspect of activation domain function is to decrease the inhibitory effect of these nucleosomes on elongation by RNA polymerase.

## **RESULTS**

### **A transcriptional pause is centered at +45 on the human *hsp70* gene in vivo.**

Previous work has identified a regulated promoter-proximal transcriptional pause at several mammalian and *Drosophila* loci (reviewed in Spencer and Groudine, 1990). In particular, on the *Drosophila hsp70* gene, paused polymerase molecules have been mapped in vivo by a variety of methods to between +20 and +30 relative to the start of transcription (Gilmour and Lis, 1986; Rougvie and Lis, 1988; Giardina and Lis, 1992; Rasmussen and Lis, 1993). To understand how regulation of pausing might be achieved, we first determined whether the phenomenon of *hsp70* promoter-proximal pausing is conserved in humans (a result expected from the extraordinary conservation of the proteins involved among metazoans), and then established the human *hsp70* promoter as an in vitro system to examine the control of pausing. In this way, we were able to compare pausing in our human cell-free system to pausing at the same locus in human cells.

To examine transcriptional pausing on the *hsp70* gene in vivo, potassium permanganate was used to footprint RNA polymerase open transcription complexes in HeLa cells. Permanganate freely diffuses through cell membranes and modifies thymine residues of single-stranded regions of DNA; hence, it is particularly useful for detecting regions of DNA in vivo that have been pulled apart by a paused, transcribing RNA polymerase molecule (Kainz and Roberts, 1992; Sasse-Dwight and Gralla, 1989; Wang et al., 1992). Modified bases can subsequently be changed to nicks by piperidine cleavage, and cleavage products can be examined by ligation-mediated PCR (Mueller and Wold, 1989). This permanganate footprinting protocol has been used previously to

detect transcriptional pausing on the human *c-myc* and *Drosophila hsp70* genes (Krumm and Groudine, 1992; Giardina and Lis, 1992).

When growing HeLa cells were treated with permanganate, subsequent analysis of the cleavage products revealed a locus of coding-strand hypersensitivity centered at +45 compared to DNA treated with permanganate in vitro (Fig. 1A, lanes 1-2), which would not contain melted regions caused by RNA polymerase molecules. By contrast, there were only two minor hypersensitive sites at +30 and +48 on the noncoding strand (Fig. 1A, lanes 3-4). Such strand specificity is expected for footprints of transcriptionally-engaged RNA polymerase molecules, since permanganate access to the noncoding strand is inhibited by the nascent transcript or by tight binding to RNA polymerase itself. Further evidence that the observed hypersensitivities were transcription-related was provided by permanganate footprinting studies done in the presence of the transcriptional inhibitors  $\alpha$ -amanitin or actinomycin-D. When HeLa cells were treated with either of these reagents prior to permanganate footprinting, the +45 hypersensitivity was reduced (Fig. 1B, lanes 3-6). Hence, it is likely that there is a paused, transcriptionally engaged RNA polymerase molecule at approximately +45 on the human *hsp70* gene in vivo .

When human cells are heat shocked, *hsp70* transcription increases approximately 20-fold (Morimoto, 1993). Nonetheless, when heat-shocked HeLa cells were subjected to permanganate analysis, permanganate hypersensitivity in the human *hsp70* gene was similar to that seen in normally growing cells (Fig. 1C, lanes 1-2). This observation matches similar findings about the *Drosophila hsp70* gene (Giardina and Lis, 1992), and implies that RNA polymerase still pauses at this sequence following activation of the promoter; however, the pause must be less penetrant or shorter in duration to account for the increase in full length transcript.



**Nucleosome-dependent pausing in vitro mimics what is observed in human cells in vivo.**

No long pauses are observed when naked *hsp70* DNA is transcribed by RNA polymerase II in vitro (see below). Since Izban and Luse (1991) had shown that nucleosomes can enhance sequence-specific pausing, we examined whether nucleosomes cause RNA polymerase II to pause at specific positions on the human *hsp70* promoter. We used a modification of the protocol of Izban and Luse in order to examine elongation of RNA polymerase at high nucleotide concentrations on nucleosomal templates. First, four point mutations were made in a template containing the natural human *hsp70* promoter so that there were no guanosine residues in the first fifteen bases of the transcript. This modified *hsp70* template was tethered to polystyrene beads to facilitate changes of nucleotide mixes during transcription (Arias and Dynan, 1989). Transcription was then initiated using HeLa basic transcription factors (partially purified to remove contaminating nucleotides), labelled UTP, and low concentrations of ATP and CTP. Under these "G-less" conditions, templates were generated that contain RNA polymerase artificially stalled at +15 with a labelled nascent transcript. Such pre-stalling of RNA polymerase complexes prior to nucleosome assembly was necessary to avoid the inhibitory effects of nucleosomes upon initiation (Knezetic and Luse, 1986; Lorch et al., 1987; Matsui, 1987), so that we could specifically examine the effects of nucleosomes upon elongation.

After nucleotides and loosely-bound proteins were washed away from the pre-stalled RNA polymerase II complexes with 1% Sarkosyl, nucleosomes or other factors were added as desired and transcription was permitted to continue by adding back high concentrations of all four unlabeled nucleotides. By performing a timecourse following the addition of nucleotides, the extent of pausing at specific positions in the *hsp70* gene could be directly determined by visualizing the end-labelled transcripts.

On naked *hsp70* DNA transcribed by this protocol, there was no evidence of a long-lasting specific pause (Fig. 2A, lanes 1-3); but if the template was assembled into nucleosomes prior to the addition of nucleotides, RNA polymerase elongation was dramatically inhibited and almost all RNA polymerase molecules remained stopped at or before +46-49 (Fig. 2A, lanes 4-6), the location of pausing *in vivo*. Specifically, the major pause sites we observed were at +46-49 (20-50% of total counts over multiple experiments) and at +27-30 (25-50%). Quantitation of the latter pause is difficult because of its proximity to the dye front, and its significance is not currently known. Both of these pauses are stable for greater than six hours, the longest timepoint tested (data not shown).

The short transcripts that we observed could have been caused either by transcription termination or by pausing. To distinguish between these possibilities, nucleosomes were removed from reactions displaying the putative paused transcripts (e.g., lane 6) by washing with 1% Sarkosyl. Transcription was then permitted to continue by adding back all four nucleotides. At the end of this protocol, only full-length transcripts were observed (lane 7); hence the paused transcripts in Figure 2A were the result of stably paused RNA polymerase that could elongate after removal of the nucleosomes and were not caused by premature termination.

### **Promoter-proximal areas of the *hsp70* transcribed region are nucleosomal *in vitro* and *in vivo*.**

To further support this nucleosome-dependent model for *hsp70* pausing, we next addressed whether templates that contained the paused polymerase were actually assembled into nucleosomes. Nucleosomal DNA is refractory to cleavage by restriction enzymes such as *Bam*HI, so if nucleosomes were required for the pause at +46-49, then these templates should not contain an accessible *Bam*HI site at +150. When artificially stalled, unassembled *hsp70* templates were cleaved with *Bam*HI, the templates were cut at +150 so subsequent elongation yielded nearly all

short transcripts (Fig. 2B, lane 1). This control confirmed the accessibility of the *Bam*HI restriction site in the absence of nucleosomes. When the templates were assembled into nucleosomes and then treated with *Bam*HI, then almost all transcripts were paused at or before +46-+49, as discussed above (Fig. 2B, lane 2). A small fraction (10%) reached the *Bam*HI site at +150, suggesting that they did not pause because they were unassembled. If these assembled and digested reactions were stripped of nucleosomes, all paused transcripts elongated to full-length transcripts (lane 3), while the background of short, unassembled transcripts remained constant at 10%. Therefore, all paused transcripts must have arisen from assembled templates. [This procedure was adapted from that described by Morse (1989).] The same experiment was conducted using restriction enzymes that cleave at various points along the template with similar results (Fig. 2C). Assembled templates were resistant to cleavage and gave full length transcripts upon removal of the nucleosomes (lanes 1,3,5,7). Unassembled templates were cut to give almost exclusively short transcripts (lanes 2,4,8), with the exception of those treated with *Pst*I, which also cleaves upstream of the start site thereby eliminating the transcript (lane 6). Digestion with *Nhe*I at +14 showed no cleavage in either the assembled or the unassembled case, as we expected because the polymerase complex is initially stalled at +15 (lanes 9,10). From these experiments, it was concluded that all transcribed templates contain a nucleosome downstream of the start site.

We next examined whether the corresponding region of the *hsp70* gene is nucleosomal in vivo. When DNA from Hela nuclei treated with micrococcal nuclease was transferred to nylon membrane and probed with a fragment of the *hsp70* gene stretching from +150 to +235, a ladder of bands with the characteristic spacing of nucleosomes was observed (Figure 2D). This pattern was not observed on naked DNA, and no signal was observed on an identical blot of DNA from micrococcal nuclease-treated mouse nuclei (data not shown), implying that the observed pattern is not an artifact either of the intrinsic susceptibility of *hsp70* DNA to micrococcal nuclease cleavage or of hybridization specificity. We have not detected specific nucleosome positioning in this region as measured by indirect end-labelling (data not shown).

### The pause observed on *hsp70* in vitro is promoter-dependent

To demonstrate that the pausing we measured in vitro was specific to the *hsp70* gene, we tested the adenovirus major late promoter in a similar in vitro assay. When this promoter instead of the *hsp70* promoter was transcribed with the protocol of Figure 2, a general inhibition of elongation and enhancement of sequence-specific pausing was observed (Izban and Luse, 1991; Figure 3A, lane 5); but there was no single locus of sharply-defined pausing like that on the *hsp70* gene (Figure 3A, lane 1). Hence, the pausing at +46-49 was unique to the *hsp70* gene.

Next, we carried out a variety of controls to address the possibility that the pause observed was of merely fortuitous length. We were concerned that the length of the linear template used in the protocol would cause nucleosomes to become positioned in a manner that would determine the location of the pause site. We found, however, that the pausing observed on *hsp70* in vitro was independent of template length: the same +46-49 pause was observed on a mixture of templates containing from 335-395 bp. of downstream sequence (Figure 3A, lane 3). Next, the independence of +46-49 pausing from the location of initial artificial stalling at +15 was demonstrated by examining pausing while varying the location of the artificial stall. We obtained the same results if RNA polymerase was stalled at +1 prior to nucleosome assembly (by initially withholding nucleotides entirely), as we did if we permitted RNA polymerase to progress to +15 prior to assembly (by adding three nucleotides) (Figure 3B). Taken together, these experiments show that the specific nucleosome-dependent pausing that we observe on *hsp70* in vitro arises from some quality intrinsic to the *hsp70* gene rather than from some aspect of our in vitro system.

### Activator-dependent release of pausing

Activation of the human *hsp70* gene following heat shock is caused by binding of an activated form of human HSF1 to the heat shock element. The transcriptional activation domains of human HSF1 (amino acids 202-529; Green et al., 1995; Zuo et al., 1995) were therefore tested for their ability to relieve pausing on the *hsp70* gene in vitro. We used a GAL4 DNA-binding domain to tether the HSF1 activation domains to the promoter region because the magnesium and detergent requirements for DNA-binding by intact HSF1 purified from human cells are incompatible with the experimental protocol used here.

When GAL4-HSF was prebound to an *hsp70* promoter containing 5 GAL4 DNA-binding sites, there was some increased readthrough of the +46-49 pause (Fig. 4A, compare the amount of paused transcript to full length transcript in lanes 1 and 2). Since GAL4-HSF addition alone did not promote a large amount of readthrough, a search for accessory factors was undertaken. One possible class of accessory factors are elongation factors known to relieve pausing in other *in-vitro* systems, such as TFIIF (Flores et al., 1989) and TFIIS (Reinberg and Roeder, 1987; Reines et al., 1989). Alone or in the presence of GAL4-HSF, though, these two factors had no effect upon the extent of readthrough of pausing (data not shown).

Since the pause at +46-49 was nucleosome dependent, we next investigated whether fractions containing hSWI/SNF activity could effect release of the pause. The yeast SWI/SNF complex has been implicated by genetic and biochemical studies to be involved in chromatin reorganization and gene activation (reviewed in Winston and Carlson, 1992), and the purified complex has been shown to possess an ATP-dependent nucleosome-disrupting activity (Cote et al., 1994). Fractions enriched for homologous complexes from HeLa cells display similar ATP-dependent reorganizing abilities (Kwon et al., 1994; Imbalzano et al., 1994). We therefore tested fractions that contain human SWI/SNF activity to see if they would enhance relief of pausing on the *hsp70* gene. Readthrough was indeed greatly enhanced by the presence of either the human SWI/SNF "A" fraction (Fig. 4A, lane 4) or the SWI/SNF "B" fraction (data not shown), two highly enriched,

chromatographically distinct fractions that both contain human homologs of the yeast SWI2/SNF2 protein (Kwon et al., 1994; Imbalzano et al., 1994). At the same time, the amount of paused transcript decreased correspondingly (See figure legend for quantitation.) These fractions had no effect upon readthrough when an ATP analogue with a non-hydrolyzable gamma phosphate was employed during the reaction (ATP $\gamma$ S, data not shown). Interestingly, the same fractions also had no effect on pausing in the absence of activator (Fig. 4A, lane 3, and see below). Hence, the actions of both an activator and an ATP-dependent activity were required for maximal relief of nucleosome-dependent pausing.

Since readthrough of pausing required the GAL4-HSF protein regardless of any accessory factors present, we next addressed whether the HSF activation domain itself was necessary for this relief. To do this, we tested the GAL4 DNA-binding domain alone (amino acids 1-94 of the GAL4 protein) to see if it could promote a similar effect in the presence or in the absence of the hSWI/SNF fractions (Fig. 4B). Under both conditions, maximal readthrough of the pause required the HSF activation domains (compare lanes 2 and 5 to lanes 3 and 6). Data from these and other experiments were quantified by determining the ratio (full length transcript)/(+46-49 paused transcript + full length transcript) using a phosphorimager, and this ratio was plotted at different concentrations of hSWI/SNF in the reaction (Fig. 5). There was no significant effect of hSWI/SNF fractions on readthrough in the absence of an activator at any concentration. Maximal effects on readthrough required both GAL4-HSF and the hSWI/SNF fraction; under these conditions readthrough increased 10-fold over that seen without activator. GAL4(1-94) had a slight effect on readthrough that was significantly lower than the effect of GAL4-HSF. [This small effect is consistent with the previously documented ability of GAL4(1-94) to stimulate transcription weakly on nucleosomal templates (Croston et al., 1992; Workman et al., 1991b)] We conclude that GAL4-HSF can increase readthrough of a nucleosome-dependent pause in this in vitro system.

### **GAL4-HSF and hSWI/SNF fractions do not affect pausing on naked DNA templates**

The effect of GAL4-HSF on pausing on the nucleosomal template might reflect a general ability of this activator to effect elongation, or might be specific to regulation of elongation on nucleosomal templates. We examined elongation of transcription on the naked *hsp70* template, and detected several positions where RNA polymerase pauses transiently, including a pause at +46-49 that had a half-life of approximately three minutes (Fig. 6). We found no effect of GAL4-HSF or hSWI/SNF fractions on the extent of pausing on the naked *hsp70* promoter, and conclude that the effects we observe upon pausing are specific to nucleosomal templates.

### **DISCUSSION**

The primary conclusions from this work are that nucleosome formation greatly enhances a transcriptional pause on the human *hsp70* gene (Figs. 2 and 3) and that the HSF1 transcriptional activation domain can relieve this pause (Fig. 4). The effect of the activation domain on pausing is seen only on a nucleosomal template; therefore, our data implies that a novel and important aspect of activation domain function is to decrease the inhibitory effect of nucleosomes on elongation of RNA polymerase. Previous studies have demonstrated that total transcription is stimulated to a significantly greater extent by activation domains on nucleosomal templates as compared to naked templates (Workman et al., 1991b; Croston et al., 1992). This difference has been attributed to an ability of activation domains to alleviate a nucleosome-dependent inhibition of pre-initiation complex formation (reviewed in Workman and Buchman, 1993). The ability of activation domains characterized here to alleviate nucleosome-dependent inhibition of elongation complements this work.

**Nucleosomes affect pausing on the human HSP70 gene**

It has been appreciated for some time that there are significant steric problems to be overcome during elongation of RNA polymerase through a nucleosome. RNA polymerase II has been shown to transcribe through nucleosomal DNA in vitro (Lorch and Kornberg, 1987; reviewed in van Holde et al., 1992); however, studies with SP6 polymerase suggest that the nucleosome is displaced in a process that appears to involve direct transfer to different DNA sequences on the same template (Clark and Felsenfeld, 1992; Studitsky et al., 1994). From these data, it seemed reasonable that nucleosomes might inhibit the rate of transcriptional elongation; and in fact nucleosome formation has been shown to increase pausing of RNA polymerase II in vitro (Izban and Luse, 1991).

We show here that nucleosome formation has a particularly dramatic effect upon formation of a paused polymerase at nucleotide +46 of the human *hsp70* transcribed region. There is a pause that lasts about three minutes at this site on naked DNA (Fig. 6). On nucleosomal templates, this pause lasts for at least six hours (the longest timepoint we have examined), and thus the length of this pause is increased at least a hundredfold by formation of the template into nucleosomes. We have used permanganate footprinting to show that a pause occurs at this same location in vivo (Fig. 1). This region of the human *hsp70* template is nucleosomal in vivo (Fig. 2), consistent with the hypothesis that, in vivo as well as in vitro, nucleosomes might contribute to formation of this pause. Such nucleosomes need not even be precisely positioned in order to facilitate specific pausing. Izban and Luse (1991) and O'Neill et al. (1992) have found that nucleosomes greatly enhance intrinsic DNA pausing sites. Studitsky et al. (1995) have reached similar conclusions, although they believe that this enhancement is confined to a particular region of the nucleosomal core. Our data concerning enhancement of the 46-49 pause is consistent with this previous work. Furthermore, pausing on the *hsp70* gene is conserved between humans and *Drosophila*, and promoter-proximal pauses are seen on several other mammalian and *Drosophila* genes (Spencer



and Groudine, 1990; Rougvie and Lis, 1988). This fact raises the possibility that the observations we have made here concerning the role of nucleosomes on inducing pauses might be more general.

### **Relief of pausing by activation domains**

Numerous studies in *Drosophila* and in mammalian tissue culture have led to the hypothesis that activators might regulate pausing of RNA polymerase II. For example, characterization of RNA polymerase occupancy of the *Drosophila hsp70* promoter demonstrated that a polymerase complex is paused proximal to the promoter prior to induction, and that induction must cause some release of the pause (Rougvie and Lis, 1988). Recently, it has been shown that activators or enhancers can decrease pausing on transfected or injected promoters (Yankulov et al., 1994; Krumm et al., 1995).

The data presented here demonstrate an effect of activators on pausing in vitro, and imply that chromatin structure plays an important role in this regulation: nucleosome formation creates an increased block to transcriptional elongation, and activation domains are able to suppress this block. Activators might relieve the block to elongation through interaction with the general transcription machinery, through a direct destabilization of nucleosomal structure in transcribed regions, or via both mechanisms. It has been proposed that promoter-proximal pausing like that observed on *hsp70* is caused by contacts between the elongating RNA polymerase and general transcription factors that remain bound to the TATA box (Usheva et al., 1992; Lis and Wu, 1993). It is possible that in the system described here, such contacts are necessary for pausing, but that the additional constraint imposed by a nucleosome is also required. In this scenario, the effect of the activator on the contacts between the general transcription factors and RNA polymerase suffices to increase elongation; however, maximal effects on nucleosomal templates require additional activities that are present in the hSWI/SNF fractions. It is unlikely that these activities are simply

facilitating activator occupancy because vast excesses of activator cannot suppress the enhancement of elongation afforded by the hSWI/SNF fraction (data not shown).

### **A possible role for nucleosome-disrupting activities in regulation of pausing**

It was necessary to supplement our in vitro system with fractions that have hSWI/SNF activity in order to achieve maximal effects of the activator on elongation. These fractions have been shown previously to contain an ATP-dependent nucleosome disrupting activity (Imbalzano et al., 1994; Kwon et al., 1994). While the effect of these fractions on elongation requires hydrolyzable ATP, and two separate highly enriched fractions (hSWI/SNF A and B) both enhance elongation, human SWI/SNF fractions are not homogeneous so we cannot rigorously conclude that the nucleosome disruption activity itself is responsible for the effects on elongation. We can rule out the possibility that previously characterized elongation factors are responsible for the relief of pausing, though, because the effects of the hSWI/SNF fractions on elongation require hydrolyzable ATP (data not shown) and there is no effect of these fractions upon elongation on naked DNA (Figure 6).

Neither of these observations is true of known elongation factors. The hSWI/SNF fractions are not sufficient to increase elongation on nucleosomal templates because they had no effect in the absence of activator (Fig. 5).

These observations are complicated further by recent evidence suggesting that there are likely to be multiple complexes with ATP-dependent nucleosome disruption activity in the cell. In human cells, there are at least three different homologs of SWI2/SNF2, the only identified component of the yeast SWI/SNF complex that contains an ATPase domain: hBrg1, hBrm, and hSNF2L (Khavari et al., 1995; Muchardt and Yaniv, 1993; Okabe et al., 1992). The former two of these proteins are both present in both of the hSWI/SNF fractions used here. The latter protein is a homolog of *Drosophila* iSWI, which is a member of the *Drosophila* NURF complex (Tsukiyama et al., 1995). The NURF complex, like hSWI/SNF, is capable of disrupting nucleosome structure in

an ATP-dependent manner, however NURF has been shown to act in conjunction with the GAGA factor -- for which there is as yet no human homolog -- in vitro at the *Drosophila hsp70* gene to disrupt chromatin structure. Given the increasing number of ATP-dependent nucleosome disrupting complexes and the ambiguity of their precise mechanism, it is difficult to assign definite biological roles to individual complexes. The work presented here, however, is most simply interpreted as suggesting that some remodeling activity is important to facilitate relief of nucleosome-dependent pausing.

Moreover, the results above also argue against a simple single-cause model for the regulation of promoter-proximal pausing. It is clear that nucleosomes are absolutely required to achieve pausing, yet chromatin-reorganizing factors alone are unable to release pausing; hence activators probably do more than just recruit such factors. Transcriptional activators will release the pause, yet are not sufficient to provide maximal release of the pause even when included at significantly higher levels than used in the experiments reported above (data not shown). It is most consistent with the data, therefore, to argue that activators and the activities present in the hSWI/SNF fraction are acting at different steps in a regulatory mechanism, and that both steps are necessary to achieve full release of pausing.

## **EXPERIMENTAL PROCEDURES**

### **Templates for in-vitro transcription**

All transcriptions were done using templates derived from the vectors pSAB8, pSABmix, pSAB12, or pML5-4NR. pSAB8 contains human *hsp70* sequences from -122 to +567 cloned *HindIII* to *HindIII* (-122) and *BglIII* to *BamHI* (+567) into the pUC18 polylinker. Three *hsp70* point mutations were engineered by PCR to eliminate guanosine residues between +1 and +14. (The modified sequence reads +1-TAACTCCTATCCTG-+14.) pSABmix is a mixture of

plasmids identical to pSAB8, except that they contain modified *hsp70* sequences only to +335, followed by zero, one, two, or three 20-bp. linkers of sequence GATCTGGCGTAATTCGGGTT. pSAB12 is a pUC18-based vector that contains five GAL4 17-mer binding sites (Giniger et al., 1985) joined to the human *hsp70* gene from -35 to +567. As in pSAB8, three *hsp70* point mutations were engineered by PCR to eliminate guanosine residues between +1 and +14. pML5-4NR (Izban and Luse, 1991) contains the Adenovirus major late promoter from -171 to +37 fused to four 150 bp. repeats from the bacterial CAT gene. (Again, point mutations had been engineered to eliminate all G residues between +1 and +15 .) It was provided to us by Dr. Donal Luse.

Prior to *in-vitro* transcription, these plasmids were linearized, tethered to avidin-acrylic beads 5' to the *hsp70* promoter, and cut at +445 (pSAB8, pSAB12) or +335,+355,+375, and +395 (pSABmix) or +3.1kb (pML5-4NR) to produce templates for runoff transcripts of these lengths. To accomplish this, all parent plasmids were cut with *EcoRI* (which cuts 2.6kb upstream of the *hsp70* promoter) and the resultant overhangs were filled in with dCTP, dGTP, dTTP, and biotinylated dATP (Gibco/BRL). The plasmids were then cut again with *EagI*(pSAB8, pSAB12), *SmaI*(pSABmix), or *NdeI*(pML5-4NR) to produce linear templates of the lengths indicated above. In each case, the digest fragment containing the promoter was purified from the others by spin-column chromatography with CL4B media (Pharmacia). The linear templates were then affixed to 0.4  $\mu$  avidin-acrylic beads (Idexx) at a concentration of 0.5-1 mg DNA per ml of bead solution by allowing DNA to adhere to beads overnight at room temperature.

#### **In-vitro transcription and nucleosome assembly (adapted from Izban and Luse, 1991)**

To preform RNA polymerase ternary complexes and end-label the transcripts (in Figures 2-6), we incubated 0.5 $\mu$ g bead-bound template for 1hr. at 30 degrees C in a 25 $\mu$ l reaction including 2mM MgCl<sub>2</sub>, 0.6mM ATP and CTP ("Ultrapure"; Pharmacia), 0.5 mM  $\alpha$ -<sup>32</sup>P-UTP (800 Ci/mmol;

N.E.N.), 20 u. RNAsin (Promega), and 15  $\mu$ l total of HeLa transcription factors and Buffer D. [Buffer D is 100mM KCl, 20% glycerol, 20mM HEPES pH 7.9, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF; factors included DE52-fractionated TFIIA and P11-fractionated other general transcription factors (Sumimoto et al., 1990). The amount of factors included in each reaction was empirically determined as that which gave maximal transcription on naked DNA. In Figures 2-3, 3  $\mu$ l of a P11 0.8M fraction and 2  $\mu$ l each of a P11 0.5M fraction and DE52-fractionated TFIIA were used. In Figures 4-6, a different preparation of the same three fractions was used, in amounts of 4  $\mu$ l, 3 $\mu$ l. and 3 $\mu$ l, respectively] Templates were then pelleted and washed once with 50  $\mu$ l of 0.6xBuffer D plus 1mM MgCl<sub>2</sub> and 1% Sarkosyl, and once with 50  $\mu$ l of 0.6xBuffer D plus 2mM MgCl<sub>2</sub>.

To preform preinitiation complexes at +1 rather than elongation complexes at +15 (Figure 3B), we incubated 0.5 $\mu$ g bead-bound template for 1hr. at 30 degrees C in a 25 $\mu$ l reaction including 2mM MgCl<sub>2</sub> and 15  $\mu$ l total of HeLa transcription factors and Buffer D. Templates were then pelleted and washed twice with 50  $\mu$ l of 0.6xBuffer D plus 2mM MgCl<sub>2</sub>. After nucleosome assembly (see below), the transcripts in these perinitiation complex reactions were end-labelled by incubating bead pellets for 1hr. at 30 degrees C in a 25 $\mu$ l reaction including 2mM MgCl<sub>2</sub>, 0.6mM ATP and CTP ("Ultrapure"; Pharmacia), 0.5 mM  $\alpha$ -<sup>32</sup>P-UTP (800 Ci/mmol; N.E.N.), 20 u. RNAsin (Promega), and 15  $\mu$ l Buffer D. The reactions were then washed once with 50  $\mu$ l of 0.6xBuffer D plus 2mM MgCl<sub>2</sub>.

To assemble templates into nucleosome cores after stalling transcription complexes on them (Figures 2-6), the washed reactions were resuspended in 25 $\mu$ l Buffer D plus 2mM MgCl<sub>2</sub> and 20 u. RNasin. To this, we added 50  $\mu$ l *Xenopus laevis* heat-treated assembly extract and 2  $\mu$ g purified HeLa core histones (Workman et al., 1991). The reactions were incubated 1.5 hrs. at 30 degrees C., and then spun down and washed with 50  $\mu$ l of 0.6xBuffer D plus 2mM MgCl<sub>2</sub>. [The heat-treated assembly extract (dHTE) used in this study was made as directed in Workman et

al.(1991), but was subsequently desalted over a P6DG column (Bio-Rad) to remove endogenous nucleotides. Extract was mixed with histones and preincubated at room temperature for 15' prior to use in the reactions described above.]

To continue the elongation -- either immediately after assembly (Figures 2-6), after transcript end-labelling (in the case of reactions with a stalled preinitiation complex, Figure 3B), or after restriction enzyme digestion (Figure 2B; see method below) -- washed reactions were incubated for 1hr. at 30 degrees C in a 25µl reaction including 2mM MgCl<sub>2</sub>, 0.6mM ATP, CTP, GTP, and UTP ("Ultrapure"; Pharmacia), 20 u. RNAsin (Promega), and 15 µl Buffer D (modified to contain 250mM KCl, so that the final KCl concentration is 150mM to inhibit reinitiation). The reactions were stopped with 50 µl STOP solution (67mM NaOAc pH 5, 6.7mM EDTA, 0.33% SDS, 0.66mg/ml tRNA).

This basic protocol was modified slightly for reactions containing activators or hSWI/SNF fractions. The modifications are described below.

Completed reactions were subjected to one extraction with phenol:CHCl<sub>3</sub>:isoamyl alcohol (25:24:1) and two precipitations with 0.3M NaOAc and 2.5 volumes EtOH. They were then analyzed on a 7.5% acrylamide/7M urea/1xTBE sequencing gel. End-labelled marker DNA (labelled M in figures) was either MspI-digested pBR322 DNA (N.E.Biolabs) or Boehringer-Mannheim Marker V. Gels were exposed and quantitated on a Molecular Dynamics phosphorimager.

### **Restriction test for template assembly (Morse, 1989); Figure 2B**

Transcription reactions were begun by transcription complex assembly followed by nucleosome assembly, exactly as described above. They were then resuspended in 25 µl Digestion Buffer

(0.6xBuffer D with 7mM MgCl<sub>2</sub>), and 20 units of the appropriate restriction enzyme (from N.E.B.) was added. Reactions were digested for two hours at 30 degrees C, and then washed once with 0.6xBuffer D plus 1% Sarkosyl and 1mM MgCl<sub>2</sub> (a treatment that also strips nucleosomes) and once with 0.6xBuffer D plus 2mM MgCl<sub>2</sub>. Elongation was then continued normally, as above; and reactions were stopped and analyzed as described previously.

### **Activators and hSWI/SNF in in-vitro transcriptions**

The GAL4 DNA-binding domain (amino acids 1-94) was purified from E. Coli as described in Chasman et al. (1989). It was 17  $\mu$ M in concentration of dimer active for DNA-binding, and was 80% pure and 80% active relative to total protein. The GAL4-HSF protein contains amino acids 1-147 of the GAL4 DNA-binding domain fused to amino acids 202 to 529 of human Heat Shock Factor 1 (characterized in vivo as the regulatory and activation domains by Green et al., 1995; Zuo et al., 1995). It was expressed in E. Coli as a 6xHis-tagged fusion with the aid of the pRJR1 expression vector (Reece et al., 1993) and purified over a nickel-sepharose column (Qiagen) according to the manufacturer's directions. The resulting preparation was 4  $\mu$ M in concentration of active dimer, and was 30% pure and 90% active. Both proteins were dialyzed into Buffer D.

Reactions containing GAL4 or GAL4-HSF (Figures 4-6) were begun by prebinding the proteins to their cognate DNA sites: we incubated 0.5 $\mu$ g bead-bound template for 15 minutes at room temperature in an 8 $\mu$ l reaction including 2mM MgCl<sub>2</sub> and a total of 5  $\mu$ l of Buffer D plus a 1.2 molar excess of activator relative to DNA-binding sites, or 1.4 pmoles. This reaction was then increased to 25  $\mu$ l in volume by supplementing with 0.6mM ATP and CTP ("Ultrapure"; Pharmacia), 0.5 mM  $\alpha$ -<sup>32</sup>P-UTP (800 Ci/mmol; N.E.N.), 20 u. RNAsin (Promega), and 10  $\mu$ l total of HeLa transcription factors and Buffer D, plus MgCl<sub>2</sub> to maintain a concentration of 2 mM, and incubated for 1 hr. at 30 degrees C. Reactions were subsequently washed, assembled, and

elongated as described above; but after each washing step reactions were supplemented with fresh protein at the same molar excess.

hSWI/SNF protein used in these studies (Figures 4-6) was purified as described in Kwon et al. (1994) as far as the EconoQ column (Pharmacia), and dialyzed into Buffer D. In these studies, it was added after assembly and prior to final elongation. Specifically, after nucleosome assembly and washing, transcription reactions were resuspended in a 23 $\mu$ l reaction including 2mM MgCl<sub>2</sub>, 4mM ATP, 20 u. RNasin (Promega), and 15  $\mu$ l total of Buffer D (modified to contain enough KCl that the concentration in the final reaction is 150mM to inhibit reinitiation) plus the amount of hSWI/SNF fraction indicated in the figure. Reactions were then incubated for 20 minutes at 30 degrees, whereupon they were supplemented with 2  $\mu$ l of the other three nucleotides (to 0.6mM.concentration). Subsequent transcriptional elongation and gel analysis of transcription products proceeded as described above.

### **Permanganate footprinting**

50 ml. of log-phase HeLa spinner cells were harvested, washed with PBS pH 7.4, and resuspended in 1 ml PBS to a final concentration of  $1 \times 10^7$  cells/ml. One-tenth volume of fresh 0.2M KMnO<sub>4</sub> was added, and the reaction was incubated for 2 minutes at room temperature. Reactions were quenched with 1.5 volumes LYSIS SOLUTION (100mM NaCl, 10 mM Tris pH 7.8, 25mM EDTA, 0.5% SDS, 1M b-mercaptoethanol, 200mg/ml proteinase K) and incubated 4 hrs. at 50 degrees C. They were then deproteinized by extraction once with equilibrated phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform; and NaOAc was added to a final concentration of 0.3M.

Alternatively, genomic DNA (obtained by lysis and deproteinization as described in the previous paragraph, but without permanganate treatment) was resuspended at 1 mg/ml. One-tenth volume



of fresh 0.2M KMnO<sub>4</sub> was added, and the reaction was incubated for 2 minutes at room temperature. The reaction was quenched with 0.5 vol DMS STOP SOLUTION (1.5M NaOAc pH 7, 1M  $\beta$ -mercaptoethanol).

In experiments in which heat-shocked cells were examined, log-phase Hela cells were incubated at 43 degrees for one hour. Cells were then washed with prewarmed PBS 7.4, and analyzed by the same protocol as cells grown at control temperature (37 degrees).

In-vivo and in-vitro reactions were both precipitated once with ethanol, washed with 70% EtOH, and briefly dried. They were then resuspended in 0.3 ml 10% piperidine, incubated 30' at 90 degrees C., and lyophilized. Dried reactions were resuspended in 100  $\mu$ l H<sub>2</sub>O and lyophilized again twice more, and then precipitated three times with 0.3M NaOAc and 2.5 vol. EtOH before a final resuspension in TE at approximately 2 mg/ml. DNA.

Cleavages were visualized by ligation-mediated PCR. LMPCR was performed according to the method of Mueller and Wold (1989), with 6  $\mu$ g of genomic DNA per LMPCR reaction. Primers used to visualize the coding strand were: Primer 1, 24-mer from +186 to +163; Primer 2, 26-mer from +154 to +129; Primer 3, 27-mer from +147 to +121 relative to the start of transcription. Primers used to visualize the noncoding strand were: Primer 1, 23-mer from -106 to -84; Primer 2, 28-mer from -73 to -46; Primer 3, 28-mer from -65 to -38.

### **Southern hybridization**

Nine plates of semi-confluent Hela cells were trypsinized from their plates, washed in PBS, and resuspended in 20 ml Buffer L (5mM PIPES pH 8, 85mM KCl, 1mM CaCl<sub>2</sub>, 5% sucrose) with 0.5% NP-40, and incubated 10 minutes on ice. Resultant nuclei were washed twice in detergent-free Buffer L, and then resuspended in 2 ml. Buffer M (15mM Tris pH 7.5, 15mM NaCl, 60mM

KCl, 15mM 2-mercaptoethanol, 1mM CaCl<sub>2</sub>, 3mM MgCl<sub>2</sub>, 0.34M sucrose). Nuclei were adjusted to 1mg/ml by monitoring 260 nm optical absorbance of a small amount diluted in 2M NaCl. Aliquots of nuclei (0.4 ml) were briefly warmed to 30 degrees C and treated with 30-600 u. micrococcal nuclease (Sigma) for 3'. Reactions were then quenched with 1 ml. STOP Buffer (50mM Tris pH 7.5, 150mM NaCl, 15mM EDTA, 0.3% SDS), and treated 1 hr. at 37 degrees with 50 µg RNase A (Sigma). Reactions were then extracted twice with equilibrated phenol, and precipitated twice with 0.3M NaOAc and ethanol.

Thirty micrograms of micrococcal nuclease-treated DNA was loaded into each lane of a 1.5% agarose gel, and the gel was run overnight at 2 volts/cm in the presence of 1xTBE and 0.5 µg/ml ethidium bromide. (Marker lanes included 2 µg of *MspI*-digested pBR322 DNA from N.E.B.). The gel was then denatured, renatured, and blotted to N.E.N. Genescreen membrane according to the instructions of the manufacturer. Nucleic acids were crosslinked to the membrane with ultraviolet light using a Stratalinker (from Stratagene), also according to the manufacturer's directions.

The blot was probed with approximately 10 ng. of *BamHI-SacII hsp70* restriction fragment (cut from pSAB8 and labelled to a specific activity of  $4 \times 10^9$  cpm/µg with a Boehringer/Mannheim Random Priming Kit.) Protocol I of the membrane manufacturer's instructions was followed during this procedure. The resultant labelled blot was exposed for five days on Kodak XAR film at -70 degrees C with an intensifying screen.

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**FIGURE LEGENDS**

**FIGURE 1** RNA polymerase pauses at approximately +45 on the human *hsp70* gene in vivo. A) Either intact HeLa spinner cells (lanes 2,4) or HeLa genomic DNA (lanes 1,3) was treated with potassium permanganate. Treated DNAs were amplified by ligation-mediated PCR (LMPCR) using primers designed to visualize coding strand cleavages (lanes 1,2) or non-coding strand cleavages (lanes 3,4) on the *hsp70* promoter-proximal region. The diagram at the bottom summarizes the enhanced cleavage sites that were observed. B) HeLa spinner cells were treated for two hours with 1 µg/ml (lane 3) or 5 µg/ml (lane 4) actinomycin D, or 1 µg/ml (lane 5) or 5 µg/ml (lane 6) α-amanitin, and subsequently treated with potassium permanganate. The coding strand hypersensitivity at +45 was visualized by LMPCR. Permanganate sensitivity of HeLa cells not treated with transcriptional inhibitors (lane 2) and of genomic DNA (lane 1) is shown for comparison. C) HeLa cells were heat-shocked at 43 degrees C. for 1 hour prior to permanganate treatment and LMPCR (lane 1), and coding strand hypersensitivity was compared to that in cells maintained at 37 degrees prior to permanganate treatment and LMPCR (lane 2).

**FIGURE 2** Nucleosome-dependent transcriptional pausing on the human *hsp70* gene. A) Transcription complexes were stalled at +15 on human *hsp70* template pSAB8 (containing a short G-less region from +1 to +15) by supplying only A, C, and radiolabelled U nucleotides. After being washed, the naked templates were either elongated immediately in the presence of all four unlabelled nucleotides for varying times (lanes 1-3) or assembled into nucleosomes and then elongated to give paused transcripts (lanes 4-6; the period of elongation for each reaction is specified above each lane). Stripping of nucleosomes from templates containing paused transcripts (generated exactly as in lane 6) with sarkosyl and then elongating again for one hour gave full-length transcripts (lane 7). B) Naked templates with artificially-stalled transcription complexes were either digested with *BamHI* and elongated to give almost all short transcripts (lane 1), or assembled into nucleosomes and then digested with *BamHI* and

elongated to give primarily paused transcripts (lane 2). If the assembled and digested templates (as in lane 2) were stripped of nucleosomes prior to elongation, nearly all long transcripts were observed (lane 3) demonstrating that *Bam*HI could not cut the assembled template. The ratio of short, digested transcripts to full-length, undigested ones is quantitated underneath appropriate lanes. C) Restriction enzyme accessibility tests identical to the ones in Part B were performed with *Sac*II (lanes 1,2), *Bam*HI (lanes 3,4), *Pst*I (lanes 5,6), *Sty*I (lanes 7,8), and *Nhe*I (lanes 9,10). In lanes 2,4,6,8, and 10, naked templates with artificially-stalled transcription complexes were digested with *Bam*HI and elongated to give almost all short transcripts. In lanes 1,3,5,7, and 9, naked templates with artificially-stalled transcription complexes were assembled into nucleosomes, digested with restriction enzyme, stripped of nucleosomes, and then elongated. The fraction of transcripts cut by restriction enzyme is indicated. D) DNA from HeLa nuclei treated with 0 u.(lane 5), 30 u.(lane 4), 90 u.(lane 3), 270 u.(lane 2), or 810 u.(lane 1) of micrococcal nuclease was Southern-blotted and probed with a fragment of *hsp70* DNA from +150-235. The marker lane contains pBR322 DNA digested with *Msp*I.

**FIGURE 3.** The *in-vitro* pause is promoter-dependent, but independent of template length and the location of initial artificial stalling. A) Pausing of RNA polymerase at +46-49 on nucleosomal templates (lane 1) and transcription of naked templates (lane 2) using the standard pSAB8 template exactly as in Fig. 2 is shown for comparison. A mixture of *hsp70* templates with 335-395 bp. of transcribed sequence (lanes 3,4) was subjected to the standard transcriptional pausing protocol: RNA polymerase complexes were artificially paused at +15; then the naked templates were allowed to continue elongation (lane 4), or the templates were assembled into nucleosomes and then elongated (lane 3). The AdMLP template p4NR (Izban and Luse, 1991) was used in the standard protocol (this template also contains a 15 base G-less region at the start), and nucleosome-dependent pausing was observed at a variety of positions (compare lanes 5 and 6), as noted previously by Izban and Luse (1991). In all cases, elongation was for one hour. The ratio of full-length transcript to 46-49 nt. paused transcripts is

listed for nucleosomal DNA below each relevant lane. **B)** Transcription complexes were initially stalled at +1 by withholding all nucleotides (**lane 2**) or at +15 by supplying three nucleotides (**lane 1**). Subsequent nucleosome assembly and elongation in the presence of all four nucleotides yielded mostly transcripts of 46-49 nt. or shorter. The ratio of full-length transcripts to 46-49 nt. transcripts is listed below each lane. Since transcription complexes stalled at +1 were stalled as preinitiation complexes rather than elongation complexes, the Sarkosyl washing step prior to nucleosome assembly, which would strip these preinitiation complexes, had to be omitted from the standard protocol. The lack of sarkosyl washing accounts for the increased background in lane 2 and in other unwashed reactions containing elongation complexes (data not shown).

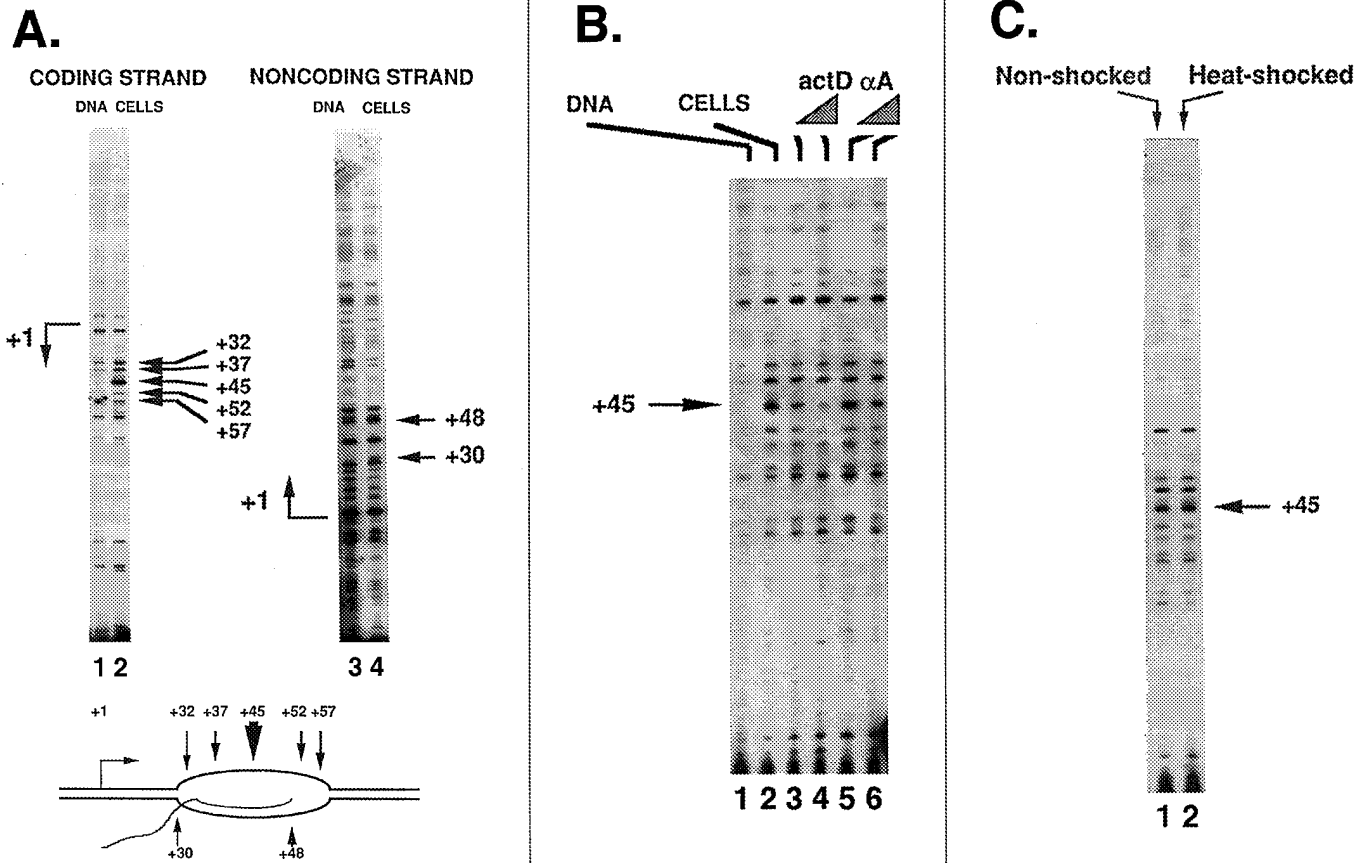
**FIGURE 4. Effects of Heat Shock Factor 1 activation domain and the hSWI/SNF fraction upon pausing.** **A)** Template pSAB12 (a *hsp70* transcription template similar to the one used in Figures 2-3 but containing 5 GAL4 DNA-binding sites) was subjected to the standard elongation protocol of artificially stalling RNA polymerase complexes at +15 by incubating template and transcription factors in the presence of A,C, and radiolabelled U nucleotides, assembling nucleosomes, and continuing elongation in the presence of all four unlabelled nucleotides (**lane 1**). In **lanes 3 and 4**, 1 unit of hSWI/SNF was preincubated with assembled templates in the presence of 4mM ATP for 15 minutes prior to the final elongation step of the reactions, and remained present during the final elongation step. In **lanes 2 and 4**, a 1.2-fold molar excess of GAL4-HSF over binding sites was preincubated with naked template for 15 minutes, and was then present during all steps of the transcription. The 46-49 nt. transcript decreases from 183 (lane 2) to 153 units (lane 4), and smaller transcripts decrease from 292 units to 242 units, for a total decrease of 80 units; the full-length transcripts increased from 32 units (lane 2) to 107 units (lane 4), for a total increase of 75 units (units are arbitrary light units as quantified by a Molecular Dynamics Phosphorimager.) **B)** Standard elongation assays (as in part A) were carried out upon pSAB12. Either no activator (**lanes 1 and 4**), the GAL4 DNA-binding domain (a.a.s 1-94; **lanes 2 and 5**), or the GAL4-HSF fusion protein (**lanes 3 and 6**) was

preincubated (at 1.2-fold molar excess over sites) with template for 15 minutes, and remained present throughout the transcription. In lanes 4-6, one unit of the hSWI/SNF fraction was preincubated with assembled templates in the presence of 4mM ATP, and remained present during the final elongation step. The hSWI/SNF fraction was absent from lanes 1-3. These experiments are quantitated in Figure 5.

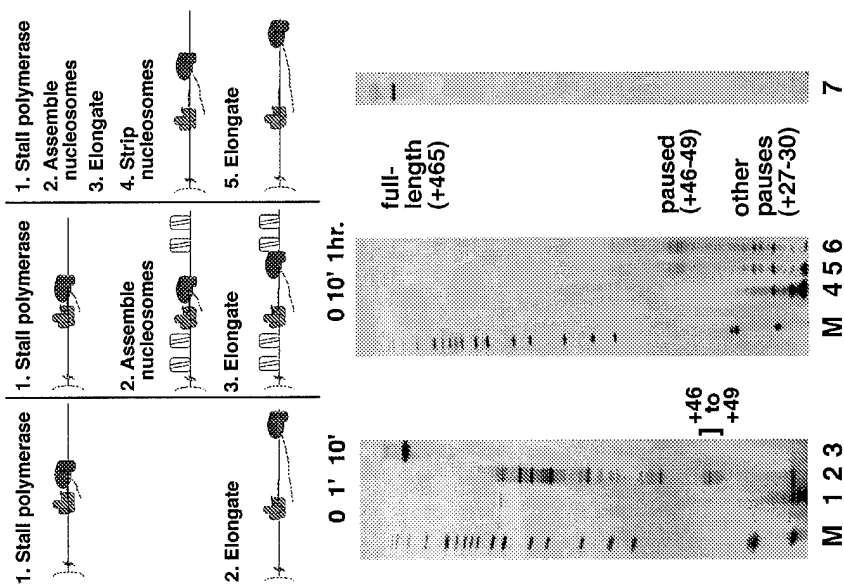
**FIGURE 5.** The effects of GAL4-HSF and the GAL4 DNA-binding domain are graphed as a function of the amount of hSWI/SNF present in the reaction. Transcriptional assays were performed exactly as in Figure 4, but with the amount of hSWI/SNF indicated along the x-axis present during the final elongation step. Readthrough was quantitated as the (amount of 465-nt. transcript)/(amount of 465-nt. transcript + amount of 46-49nt. transcript). The data shown is from a single typical titration; the trends observed were verified in several other such titrations.

**FIGURE 6.** Effects of GAL4-HSF and hSWI/SNF upon pausing on naked DNA templates. Transcription complexes were artificially stalled at +15 on pSAB12 templates by adding only A, C, and labelled U nucleotides. After washing, the complexes were elongated by adding all four unlabelled nucleotides and incubating for 0' (lanes 1,6,11,16), 1' (lanes 2,7,12,17), 3' (lanes 3,8,13,18), 10' (lanes 4,9,14,19), or 30' (lanes 5,10,15,20). In lanes 11-20, GAL4-HSF was preincubated (at a 1.2-fold molar excess over binding sites) with templates for 15 minutes prior to the start of transcription reactions, and remained present throughout the reactions. In lanes 6-10 and 16-20, one unit of the hSWI/SNF fraction was incubated with artificially stalled templates in the presence of 4mM ATP for 15 minutes prior to the addition of all four nucleotides, and remained present throughout the elongation.

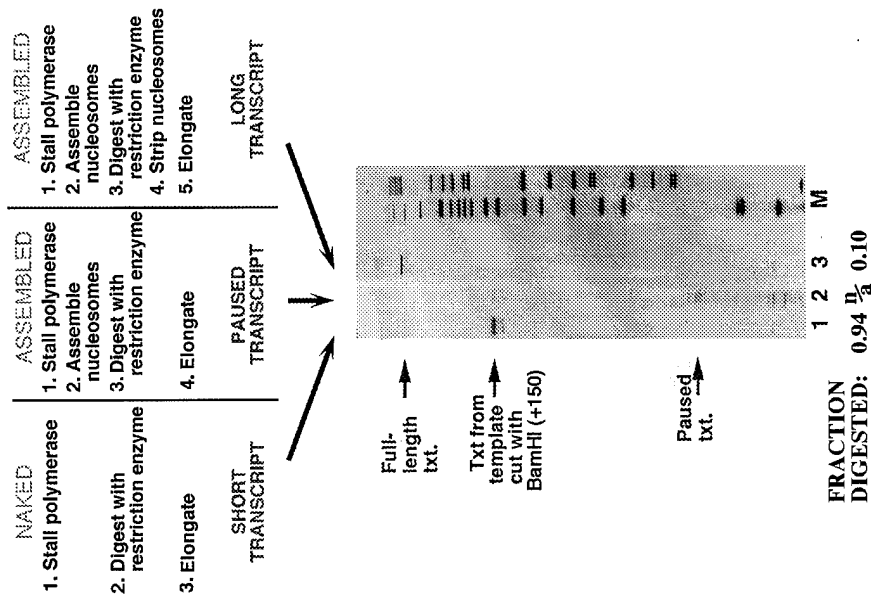
Figure 1



**A.**



**B.**





**C.**

**Digestion position:**

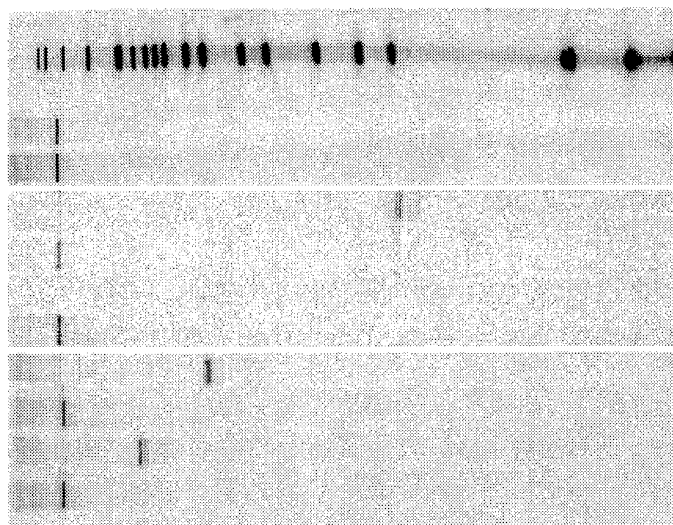
+235 +154 +101 +68 +14

**Nucleosomes:**

+ - + - + - + - + - + -

full  
length

cut

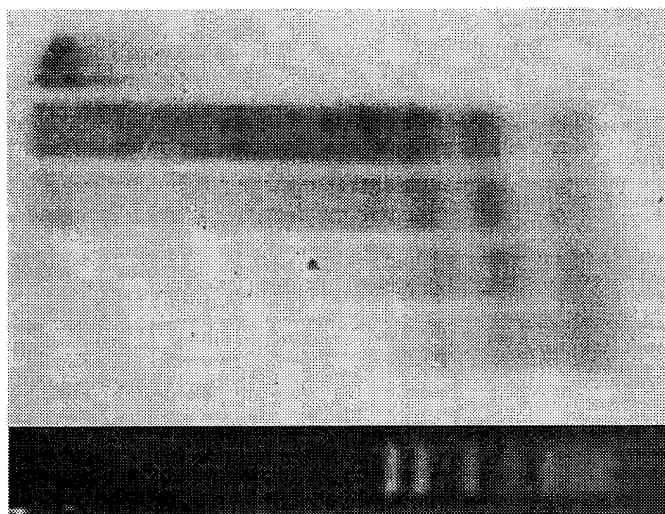


**Fraction digested**

assembled:  
naked:

1 2 3 4 5 6 7 8 9 10  
.05 .04 .03 .03 .09 .01  
.88 .90 n/a .92 .01

**D. micrococcal nuclease**



622  
527  
404  
309  
242-  
147

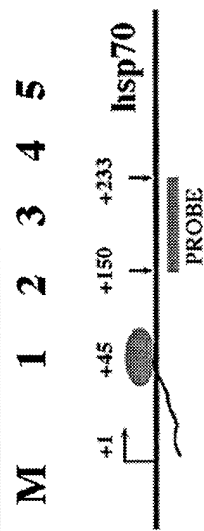
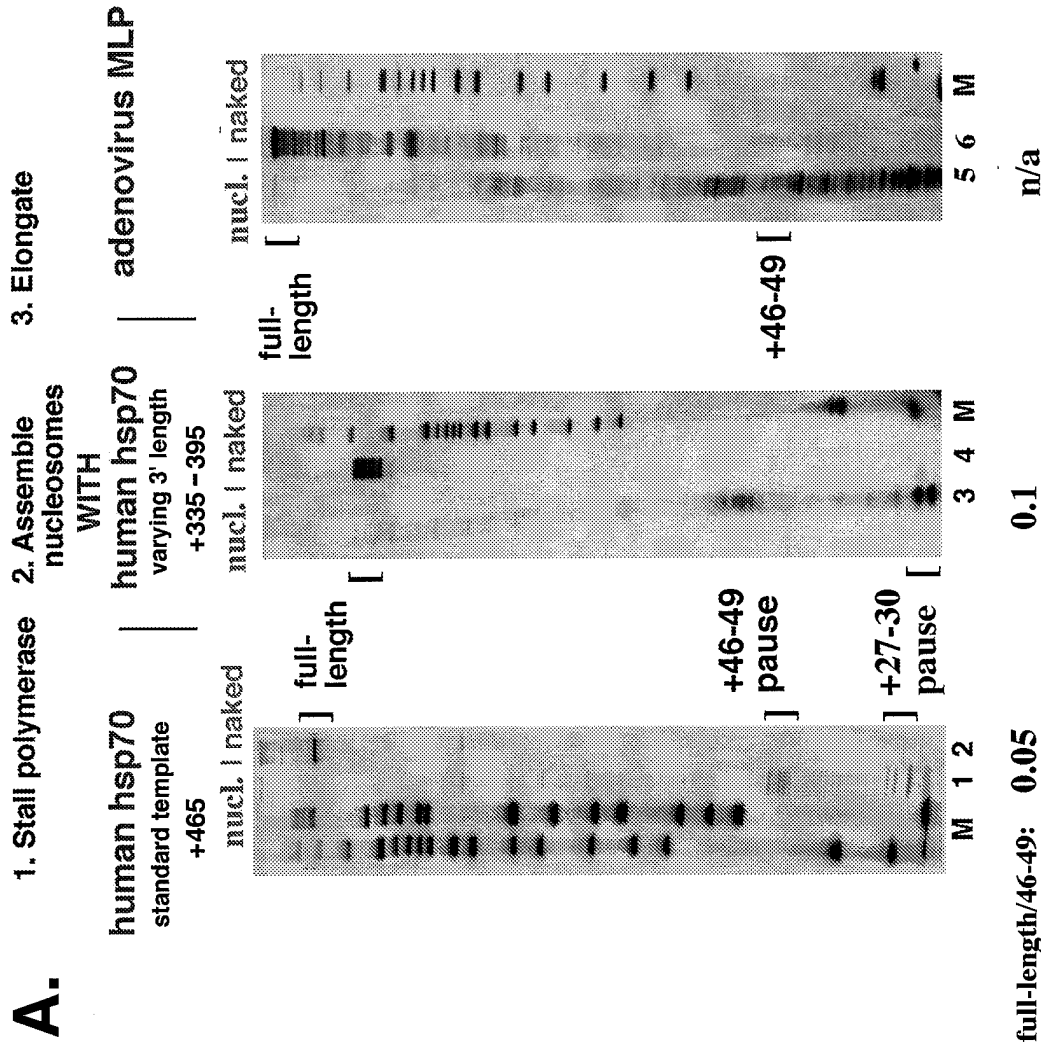


Figure 2 C & D

**A.**



**B.**

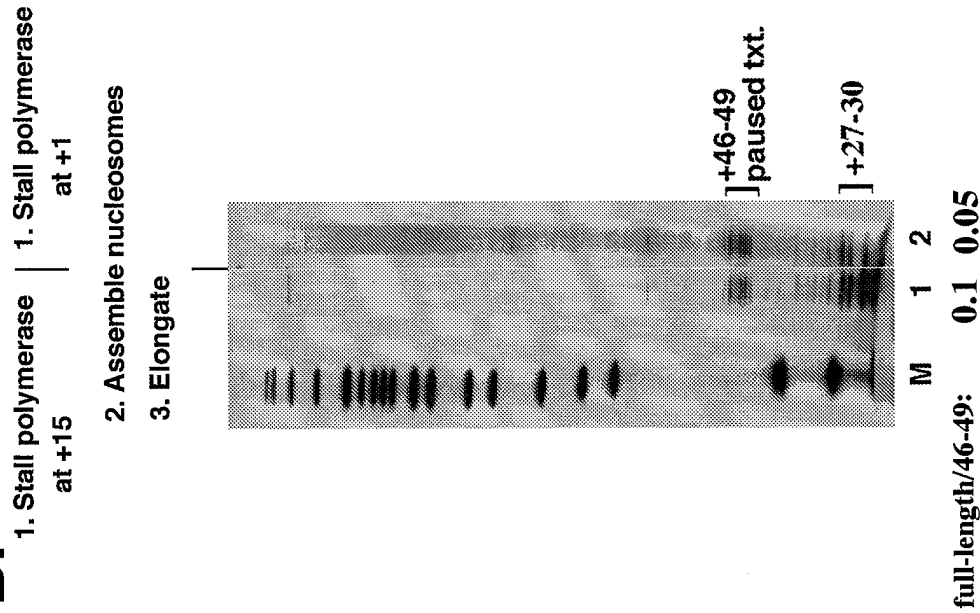
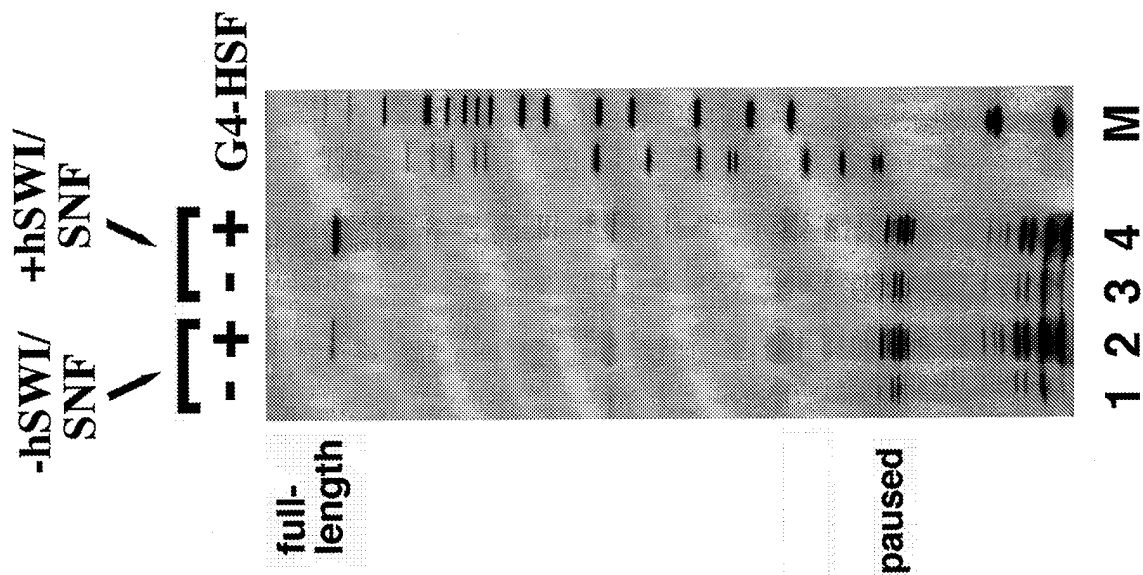


Figure 3 A & B

**A.**



**B.**

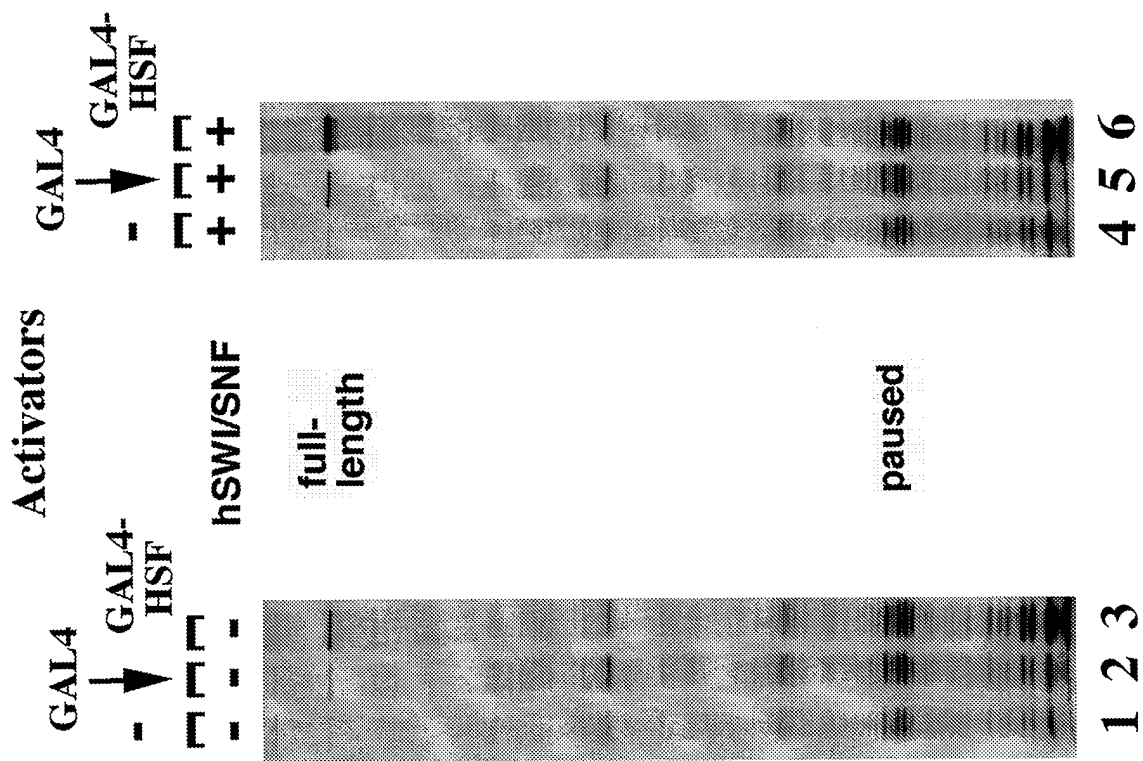


Figure 4 A & B

Figure 5

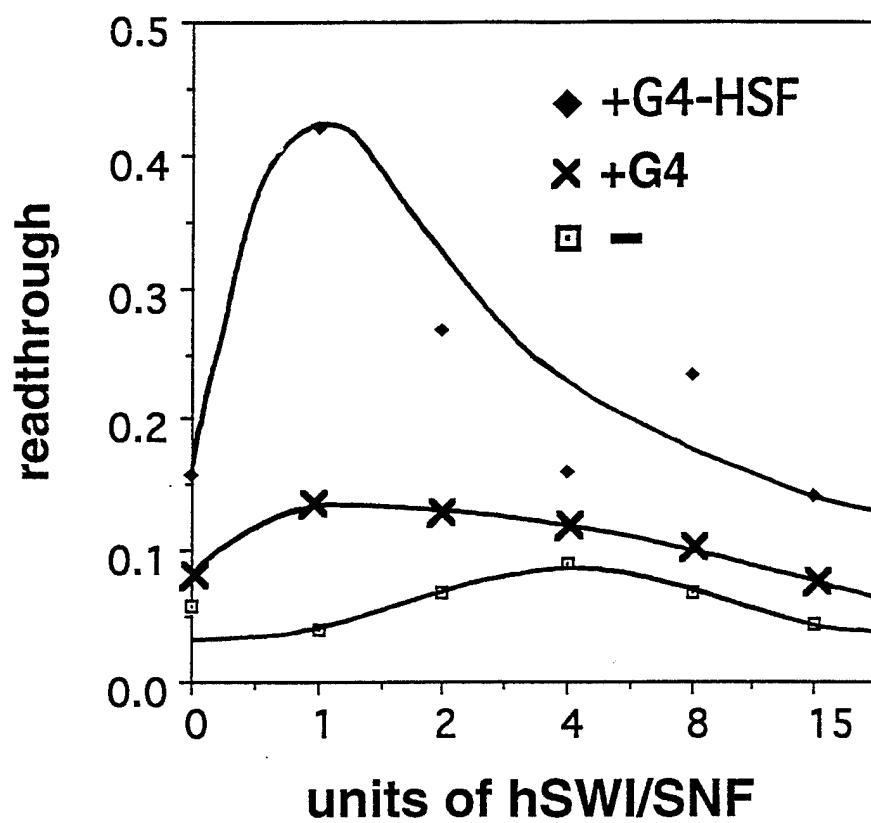


Figure 6

